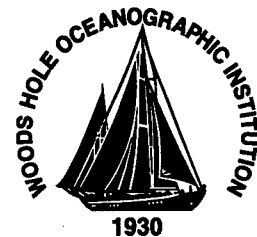


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**Massachusetts Institute of Technology  
Woods Hole Oceanographic Institution**



**Joint Program  
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**DOCTORAL DISSERTATION**

*A Molecular Approach to Questions in the Phylogeny  
of Planktonic Sarcodines*

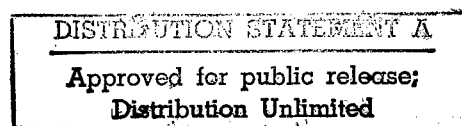
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Linda Angela Amaral Zettler

September 1996

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**A Molecular Approach to Questions in the Phylogeny  
of Planktonic Sarcodines**

by

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September 1996

**DOCTORAL DISSERTATION**

Funding was provided by: a National Science Foundation Minority Graduate Fellowship; the National Science Foundation through Grant No. OCE-9314533; the Scurlock Foundation; the Bermuda Biological Station for Research, Inc.; the Woods Hole Coastal Research Center; the Lerner Gray Fund for Marine Research of the American Museum of Natural History and the Education Office of the Woods Hole Oceanographic Institution.

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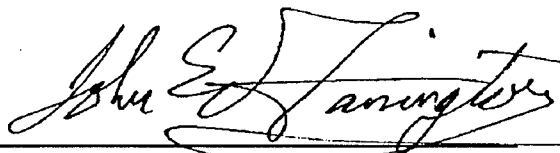
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**A Molecular Approach to Questions in the Phylogeny of Planktonic  
Sarcodines**

by

**Linda Angela Amaral Zettler**

Sc.B. Aquatic Biology

Brown University, 1990

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

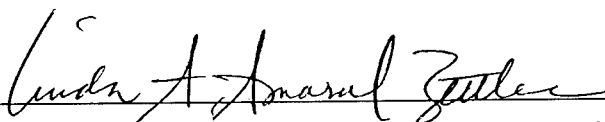
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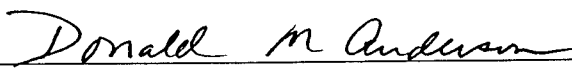
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# **A Molecular Approach to Questions in the Phylogeny of Planktonic Sarcodines**

by

**Linda Angela Amaral Zettler**

Submitted to the Department of Biology in August, 1996 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

## **Abstract**

The Acantharea and the Polycystinea are two classes of sarcodines (Sarcodina) which are exclusively planktonic and occur strictly in oligotrophic marine environments. Although these protists have been the topic of research since Ernst Haeckel's systematic investigations of samples from the H. M. S. Challenger Expedition, many aspects of their phylogeny and systematics remain poorly resolved. Part of the problem is that the criteria used in systematics of these groups until now has emphasized morphological elements which may be similar due to convergence rather than common ancestry. The application of molecular biology to the field of biological oceanography offers alternative approaches to reexamining sarcodine phylogeny with the goal of producing classifications which reflect evolutionary history.

The relationships of the Acantharea and the Polycystinea (order Spumellarida) to other protists were investigated using phylogenetic analyses of small-subunit ribosomal RNA (SSU rRNA) genes. Members of these two classes have been traditionally grouped into the common superclass Actinopoda based on their specialized pseudopodia called axopodia. Sequences from two orders of Acantharea (Symphyacanthida and Chaunacanthida) and four representatives from the order Spumellarida and the class Polycystinea (one solitary and three colonial spumellaria) were aligned against 25 other eukaryotic SSU rRNA sequences extracted from a data base of more than 800 eukaryotic sequences and subjected to distance, maximum parsimony and maximum likelihood analyses. SSU rRNA-based phylogenies do not support the common ancestry of the Acantharea and the Polycystinea, implying that the superclass Actinopoda is artificial and should be discarded. The respective monophyly of the Acantharea and the Polycystinea were supported in all analyses accomplished. The origin of the sequences was confirmed by *in situ* hybridization experiments.

SSU rRNA gene sequences for the solitary spumellarian Thalassicolla nucleata were compared from individuals collected from the Sargasso Sea and the Pacific Ocean. Sequences from pooled individuals showed primary structure differences which were consistent with genus-level variation reported in the literature for unrelated taxa. These results indicate that there may be different strains of this genus which are morphologically identical or that perhaps there may be allelic variation within a given individual.

The evolutionary relationships between the solitary T. nucleata and seven colonial spumellaria were analyzed to determine whether the two families of colonial spumellaria (Collosphaeridae and Sphaerozoidae) form a monophyletic evolutionary assemblage. Phylogenies inferred from distance and maximum likelihood methods did not support the monophyly of the colony-forming spumellaria. Parsimony methods did support the monophyly of the colonial spumellaria but with very low bootstrap support. The monophyly of members from the Collosphaeridae family was supported in all analyses with 100% bootstrap support while only distance analyses supported the monophyly of the Sphaerozoidae. The possibility that coloniality has evolved more than once in the Spumellarida has been suggested from observations of the fossil record. However, contrary conclusions have been reached from studies based on skeletal morphogenesis. The results obtained from molecular analyses question the utility of coloniality as a reliable phylogenetic marker. Sequence variation within the SSU rRNA genes of the Spumellarida appears to be sufficient enough for continued fine-scaled comparisons between existing morphospecies.

The branching patterns within three of the four orders of the Acantharea were examined using additional SSU rRNA gene sequence data from representatives of the Symphyacanthida, Chaunacanthida and Arthracanthida. The results from this analysis revealed a phylogeny which placed one representative of the Symphyacanthida (Haliommatidium sp.) branching among the Arthracanthida. An examination of the cytological features of Haliommatidium sp. in the literature revealed morphological similarities it shares with the Arthracanthida that could corroborate this result. The variability within acantharian SSU rDNA was significantly less than that observed in spumellaria, and may prove less useful in establishing relationships at taxonomic categories below the order level.

Name and Title of Thesis Advisor: David A. Caron, Associate Scientist with tenure

This dissertation is dedicated to my grandmother,  
Guisepina Quattrocchi Cavallaro  
1902-1987  
whose  
formal education ended after  
first grade, when an earthquake destroyed her school.





## Acknowledgments

I am sincerely indebted to the many people who made the work accomplished during this dissertation not only possible but also very enjoyable over the past five years. Perhaps the person who has had the most influence on my academic career has been my advisor David Caron. My first introduction to Woods Hole and WHOI were largely the result of a desire to work with Dave, a world expert on the biology of planktonic sarcodines, and learn more about a group of protists for which I had, until that point, studied only the fossil remains. I am grateful for his guidance and enthusiasm to allow me to pursue a research topic peripheral to other projects in his lab.

I am also grateful to my committee members Mitch Sogin, Richard Norris, Paul Matsudaira and John Waterbury (Chairperson) for their guidance over the past five years. I need to especially acknowledge Mitch Sogin who allowed me to conduct part of my research in his lab during the early stages of my work. Members of his lab, especially Greg Hinkle went above and beyond the call of duty to assist me in various aspects of molecular biology and phylogenetic analyses. I am also thankful to Hilary Morrison and Jeff Silberman, also of the Sogin lab. Both have answered countless questions and Hilary especially, has served as a model of how work efficiently in a lab.

Although members of the Caron Lab have turned over several times during my stay at WHOI, Elaine (Ee Lin) Lim has been around for the duration. From trips to Long Island in search of the "Brown Tide" to countless late nights in the lab in search of nothing but slumber, Elaine and I have shared many experiences which will not soon be forgotten. I am grateful for her role as teacher and fellow labmate, officemate and friend. Newer members of the Caron lab have also provided valuable assistance: Mark Dennett has spent time assisting me in perpetual computer-related questions as well as other topics too numerous to mention and Becky Gast has contributed to both the field and lab components of my work and was kind enough to read parts of the drafts of this thesis. The remaining members of the Caron lab : Ewann Agenbroad, Mike Atkins, Katie Boissonneault, Per Carlsson, Dawn Moran, and Jeff Shimeta should all be thanked for their support during the final throws of my dissertation preparation. Other WHOI affiliates who have been of support include Gaspar Taroncher, John Kokinos, Alexi Shalapyonok, Diana Franks, Judy McDowell, Jake Peirson and many others. I also need to mention people outside of WHOI: Ed DeLong (UCSB) served as my first mentor in molecular techniques, Annika Sanfilippo and William Riedel (Scripps) shared their radiolarian bibliography with me, Neil Swanberg (IGBP) assisted in taxonomic identifications of radiolaria, and O. Roger

Anderson also assisted in radiolarian identifications and took part in numerous e-mail and telephone conversations regarding aspects of radiolarian biology.

A very special thanks to the scientific and administrative staffs at the Bermuda Biological Station for Research, Inc., (BBSR) where I conducted the bulk of my field work. Tony Michaels not only assisted in acantharian identifications but also opened up his lab during my numerous visits to the station. Tony greatly assisted both personally collecting sarcodines and also allowing members of his lab especially Frances Howes to participate in blue water diving collections. I also thank Hank Trapido-Rosenthal for allowing me to use his molecular equipment while I was conducting experiments at the station. Members of the crews of the R/V Weatherbird and R/V Henry Stommel based at BBSR have made voyages out into the deep blue Sargasso pleasurable ones.

I especially want to acknowledge my family. My parents have always made education a very high priority in their lives and I am grateful for the sacrifices they have endured on my behalf. Finally, my husband and best friend Erik Zettler has been a continuous source of encouragement and support for the duration of my dissertation and it is to him that I owe my deepest thanks.

This material is based upon work supported under a National Science Foundation Minority Graduate Fellowship. Funds were also provided in part by NSF (OCE-9314533) (awarded to D. A. Caron ) and by the following awarded to L. A. Amaral Zettler: funds from the Scurlock Foundation, grant-in-aid funds directly from the Bermuda Biological Station for Research, Inc., funds from the Woods Hole Coastal Research Center, the Lerner Gray Fund for Marine Research of the American Museum of Natural History and support directly from the Education Office at the Woods Hole Oceanographic Institution.

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## **Introduction**



Planktonic sarcodines are a heterogeneous group of single-celled aquatic eukaryotes which include amoebae, foraminifera, and actinopods. While many planktonic sarcodines share similar ecological niches in marine and freshwater environments, it is unclear to what extent they share a common evolutionary history. Taxonomic frameworks that encompass members of the planktonic sarcodines are equivocal. This is largely because many of the morphological characters upon which these classification schemes were built are probably not phylogenetically meaningful. Some taxonomic schemes which were created at the turn of the century have seen little revision since their creation and are still in use.

Planktonic sarcodines are grouped within the subphylum Sarcodina based on the possession of pseudopodia during some part of their life cycle. The validity of this grouping has been questioned and current opinion largely regards it as an artificial taxon (Corliss 1984; Page 1987; Cavalier-Smith 1993). Further taxonomic divisions of the Sarcodina based on specialized pseudopodial structures such as axopodia, possessed by members of the superclass Actinopoda, have also come under scrutiny (Cavalier-Smith 1993). The question of the monophyly of the Actinopoda remains largely unresolved.

The application of molecular biological techniques to protistan systematics provides an independent means of examining existing systematic frameworks based on classical approaches. This thesis considers the evolutionary relationships between and among two currently-recognized actinopod classes, the Acantharea and the Polycystinea, based on sequence analysis of small-subunit ribosomal RNA genes. The reconstruction of phylogenies based on SSU rRNA genes aims to establish the relatedness of the Acantharea and Polycystinea to other eukaryotes and ultimately to provide information for further development of "natural" classification schemes within these classes.

### **General Background on Acantharia and Polycystine Radiolaria**

Acantharea and Polycystinea are two classes of axopod-bearing protists which are strictly planktonic and found exclusively in open-ocean oligotrophic environments (see Fig.

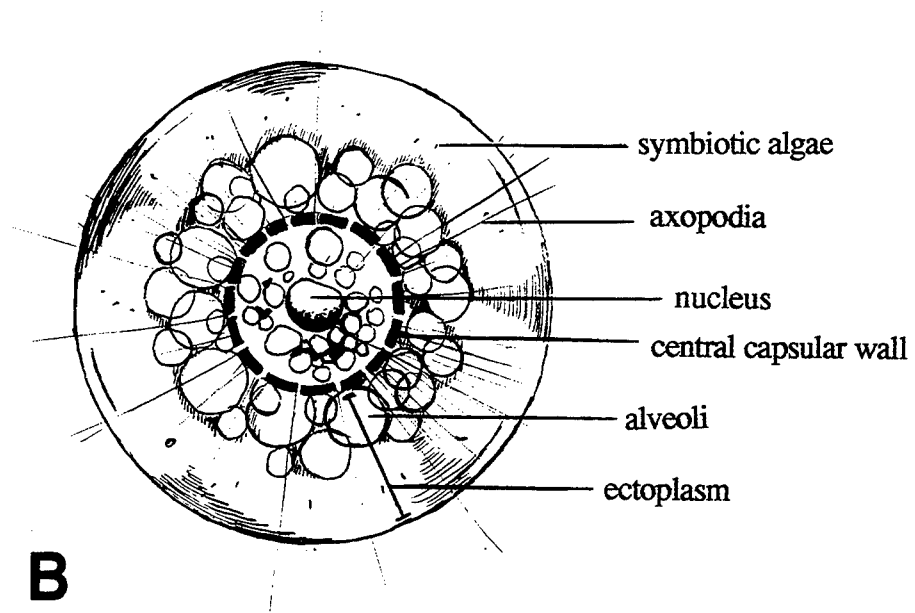
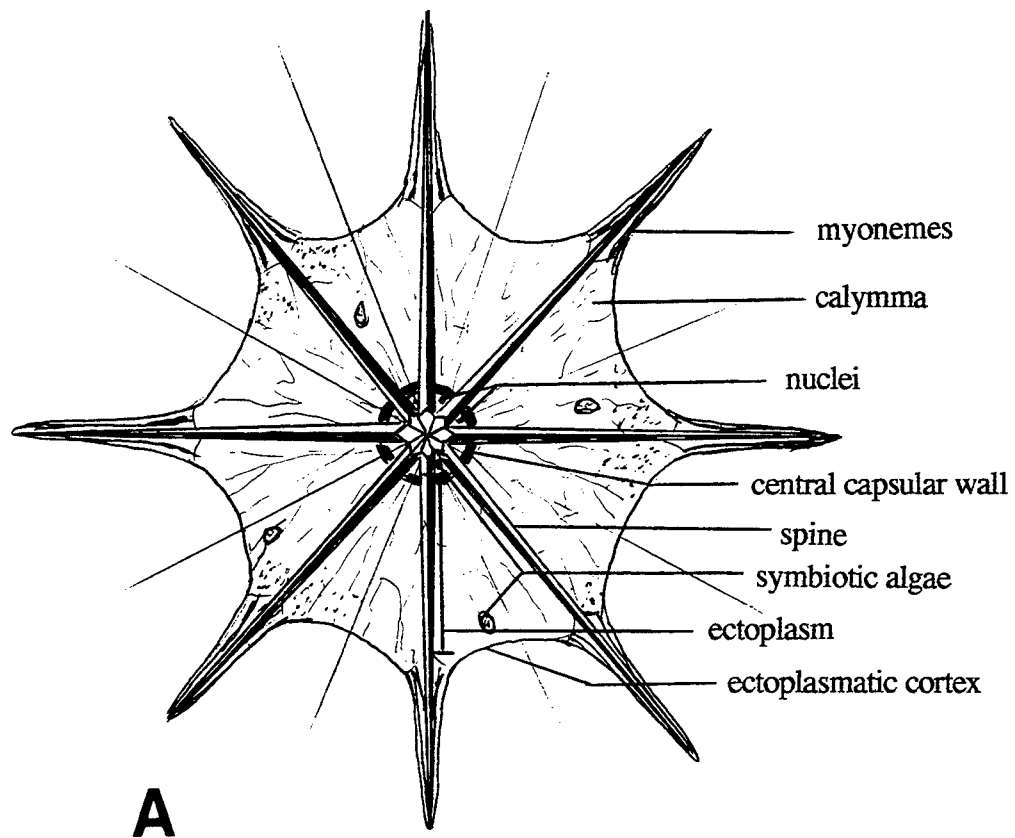
1). Among the larger planktonic sarcodines, the acantharia and particularly the colony-forming polycystines are perhaps the most conspicuous of the zooplankton found in the open ocean owing to the bloom-like conditions often created by the Acantharea and due to the conspicuous visibility of large macroscopic colonies of spumellarian members of the Polycystinea. Acantharia range in size from 50-800  $\mu\text{m}$  in diameter, solitary spumellarians range from 10  $\mu\text{m}$  to several centimeters in diameter, and colonial spumellaria have been reported up to three meters in length. Despite an often visible presence in the plankton, sampling methods and preservation techniques have led to underestimates of living acantharian and spumellarian abundances and ultimately underestimation of their importance in marine environments (Swanberg 1979; Michaels 1988).

Although living polycystines have received limited study, their fossilized skeletons have not. Many polycystines including the colonial spumellaria considered in this thesis possess siliceous skeletons which are preserved in marine sediments. The polycystines offer the longest geological and widest biogeographical ranging microfossils available for study by micropaleontologists (Casey et al. 1983). The long fossil record left behind by shell-bearing Polycystinea has precipitated a suite of research spanning paleoclimatological, paleoecological, and evolutionary studies (e. g. Riedel and Sanfilippo 1971; Kellogg and Hays 1975; Lazarus 1983).

Unlike the Polycystinea, members of the Acantharea, which possess shells of strontium sulfate, are absent from the fossil record and have thus received far less attention than the Polycystinea. Despite a lack of geological utility, the acantharia have been the topic of various kinds of biological research. Since they possess unique non-actin myonemes, they have been intensively studied by cell biologists (Febvre 1990 and the references therein). As the sole protistan utilizers of strontium sulfate as the major structural component of their skeletons, acantharia serve an important function in the strontium cycle of the world oceans (Bernstein et al. 1987). Since members of the



Fig. 1. A schematic of hypothetical **A.** acantharian and **B.** spumellarian cells indicating cell structural features of the two different sarcodines.



Acantharea and Polycystinea often live in association with symbiotic algae, they also play a role in both primary productivity and microbial food-web dynamics (Michaels 1988; Caron and Swanberg 1990; Caron et al. 1995).

The inability to culture acantharia and polycystines through successive generations in the laboratory has been an important impediment to their study. Our inability to maintain sarcodines in laboratory culture and the care which must be taken in collecting these fragile organisms has resulted in a fragmentary understanding of their biology and more fundamentally, their relationships to other organisms. However, despite recalcitrance to laboratory culturing, acantharia and polycystines have been the objects of study for over a century and a half.

#### **Historical Perspective: Haeckel's Radiolaria**

One of the first described radiolaria (Meyen 1834) was a colonial polycystine spumellarian, belonging to the spicule-bearing genus Sphaerozoum. Ehrenberg (1838) erected the first classification for the radiolaria and is credited as giving the Polycystinea their name, which was derived from descriptions of spherical latticed-shells found of fossil polycystines. Earliest reports of acantharia were made by Müller (1858) who grouped the acantharia and the radiolaria together based on a shared radial disposition of the pseudopodia possessed by both groups. The highly-ordered geometrical pattern of spine orientation formed by acantharian cells has been called Müller's Law in recognition of his early observations of acantharian skeletal architecture (see Chapter 4, Fig. 1C). Thus, from the very early stages of their study, shared morphological similarities of the Acantharea and the Polycystinea united these two sarcodine groups into a common taxonomic category.

The first exhaustive accounts of both acantharian and polycystine systematics were those of Ernst Haeckel after the H. M. S. Challenger Expedition. Haeckel (1883, 1887) combined the Acantharia (modern-day Acantharea) and the Spumellaria and the Nassellaria



(now classified collectively in the class Polycystinea) along with the fourth "legion", the Phaeodaria (Phaeodarea), into the "class" Radiolaria. He further united the Acantharia and the Spumellaria (Spumellarida) into the now defunct "subclass" Porulosida based on the shared characteristics of the distribution and size of the pores in the central capsule wall. In 1909, the Acantharea and Polycystinea were grouped along with other sarcodines based on the structure of their pseudopodia into what was first created as a "class" by Calkins called the Actinopoda (Calkins 1909). The Actinopoda, still persists in modern classifications but has been elevated to a superclass (Fig. 2). The superclass Actinopoda was originally created to encompass all sarcodines which possessed microtubule-supported pseudopodia termed axopodia.

#### **Historical Perspective: Acantharea**

Following the establishment of the Actinopoda in 1909, Schewiakoff (1926) promoted the Acantharia to the level of subclass based on the differences he noted in the capsule membrane of acantharia from the radiolaria and the absence of central capsules in certain acantharia. He also revised lower level acantharian systematics to take into account cytological features. The "subclass" Acantharia was elevated to the level of superorder by Enriques (1931). He called the new superorder "Birefrangientia" based on the birefringant properties of the strontium sulfate-containing acantharian skeleton. The current status of the "class" level of organization currently given to the Acantharea was first proposed by Tregouboff (1953) and has been accepted by other specialists since that time.

#### **Historical Perspective: Polycystinea**

The remainder of the historical review will be restricted to polycystine systematics because only a limited group of spumellarian representatives from the class Polycystinea were addressed in this thesis.

Huxley (1851) further elaborated on the work of Meyen and assigned all colonial spumellaria to the species Thalassicolla punctata. However, Müller disagreed with the



Class: Actinopoda  
 Order: Symphyacanthida  
     Family: Pseudolithidae  
         Genus: Haliommatidium sp.  
  
 Order: Chaunacanthida  
 Order: Arthracanthida  
     Suborder: Sphaenacanthina  
         Family: Acanthometridae  
             Genus: Acanthometra  
                 Acanthometra sp.  
  
 Class: Polycystinea (radiolaria)  
 Order: Spumellarida  
     Suborder: Sphaerocollina  
         Family: Thalassicollidae  
             Genus: Thalassicola  
                 Thalassicola nucleata  
  
         Family: Sphaerocoidae  
             Genus: Collozoum  
                 Collozoum pelagicum  
                 Collozoum serpentinum  
             Genus: Sphaerozoum  
                 Sphaerozoum punctatum  
             Genus: Rhaphidozoum  
                 Rhaphidozoum aciferum  
  
         Family: Collosphaeridae  
             Genus: Collosphaera  
                 Collosphaera globularis-huxleyi  
  
         Genus: Acrosphaera  
             Acrosphaera (circumtexta?)  
  
         Genus: Siphonospaera  
             Siphonospaera cyathina

Kingdom: Protista  
Phylum: Sarcomastigophora  
Subphylum: Sarcodina  
Superclass: Ac

Order: Chaunacanthida  
Order: Arthracanthida  
Suborder: Sphaenacanthina  
Family: Acanthometridae  
Genus: Acanthometra  
*Acanthometra* sp.

Class: Polycystinea (radiolaria)  
Order: Spumellarida  
Suborder: Sphaerocollina  
Family: Thalassicollidae  
Genus: Thalassicolla  
Thalassicolla nucleata  
Family: Sphaerozoidae  
Genus: Collozoum  
Collozoum pelagicum  
Collozoum serpentinum  
Genus: Sphaerozoum  
Sphaerozoum punctatum  
Genus: Rhaphidozoum  
Rhaphidozoum aciferum  
Family: Collosphaeridae  
Genus: Collosphaera  
Collosphaera globularis-  
huxleyi  
Genus: Acrosphaera  
Acrosphaera (circumtexta?)  
Genus: Siphonosphaera  
Siphonosphaera cyathina

inclusion of the colonial radiolaria within the single genus Thalassicolla which was already known to contain the solitary spumellarian T. nucleata. Müller was the first to differentiate between the solitary and colonial spumellaria which he called the Solitaria and the Polyzoa respectively (Müller 1858; Strelkov and Reshetnyak 1971).

Haeckel was the next taxonomist to substantially revise colonial spumellarian systematics based on the presence or absence of skeletal features. He described 84 species, 17 genera and 3 families. After Haeckel's 1862-1887 systematic revisions of the spumellarian polycystines, further taxonomic revisions were largely the efforts of Brandt (1885, 1905) and his students. According to Strelkov and Reshetnyak (1971), Brandt disagreed with many aspects of Haeckel's classification, most importantly of which was his separation of the colonial spumellarian radiolaria into different orders. Brandt created a separate taxon which included all colonial radiolaria into the one group called the Sphaerozoa and reduced the number of species, genera and families proposed by Haeckel. Brandt kept the two families of colonial spumellarians recognized in modern classifications, the Collosphaeridae and the Sphaerzoidae. These two families were grouped into the suborder Polycyttaria by Haecker in 1908. Haecker also divided the Spumellaria into two additional suborders, the Sphaerellaria, which contained solitary shell-bearing forms and the Collodaria which contained either skeletonless or spicule-bearing solitary forms such as Thalassicolla. Further systematic revisions were carried out by Hilmer (1906), Breckner (1906), and Popofsky (1908) later by Tregouboff (1953). The latest revision of the colonial spumellaria has been carried out by Strelkov and Reshetnyak (1971).

### **Modern Day Classifications and Taxonomic Perspectives**

Although not formally recognized, some revisions have been suggested in the recent literature to reflect a more "natural" classification scheme for the Actinopoda. Under current classification schemes recognized by the Committee in Systematics and Evolution of the Society of Protozoologists (Levine et al., 1980), the Actinopoda is a superclass

which includes four classes: the Acantharea, Polycystinea, Phaeodarea and Heliozoa. However, recognizing the diversity within the Actinopoda, Cavalier-Smith (1987) elevated the taxon Actinopoda to a "parvkingdom". He further recommended a division of the Actinopoda into the phyla "Radiozoa" and "Heliozoa" in order to account for recognized differences between the Heliozoa (Heliozoa) on the one hand and the Acantharea, Polycystinea and Phaeodarea (Radiozoa) on the other. While recognizing its diversity, Cavalier-Smith maintained the taxon Actinopoda suggesting that it might be monophyletic. More recently, Corliss (1994) adopted Cavalier-Smith's "Radiozoa" and the further divisions of the subphylum Acantharia with the class Acantharea and the subphylum Radiolaria with the classes Polycystinea and Phaeodarea.

The classification scheme used throughout this thesis does not incorporate the most recent suggestions as indicated above since no real consensus has been reached on the appropriate revisions, but instead adopts the last formally revised classification of the Protozoa made by the Committee on Systematics and Evolution of the Society of Protozoologists (Levine et al., 1980) (see Fig. 2). The Levine et al.(1980) classification scheme is used for higher level classifications wherein phyla, subphyla and superclasses end in "a"; classes end in "ea"; subclasses in "ia"; orders in "ida"; and suborders in "ina". The classification scheme of Strelkov and Reshetnyak (1971) has been used for the colonial spumellaria and that found in Lee et al. (1985) for the solitary spumellaria and the acantharia since these schemes address systematic groupings below the suborder level whereas Levine et al. (1980) stops at suborder-level classification.

As mentioned earlier, the work described herein attempts to use newly-developed molecular methods as independent tools for examining sarcodine phylogenetic relationships and producing classifications which reflect these phylogenetic relationships. There are many reasons for choosing ribosomal RNA molecules to address the phylogenetic and systematic questions posed above. These include their ubiquitous occurrence among all

living organisms, their functional uniformity, and absence of lateral gene transfer (Olsen et al. 1986; Sogin et al. 1986; Field et al. 1988). Ribosomal RNA molecules possess both very conserved and very variable regions which allow for nucleotide base pair alignments between both closely and distantly-related organisms (Gobel et al. 1987; Sogin and Gunderson 1987). In addition to these features, the current data base for rRNA gene sequences is one of the largest of its kind, and so allows for comparisons between many different organisms (Neefs et al. 1991; De Rijk et al. 1992).

Yet another advantage of rRNA-based analysis is the potential for constructing phylogenetic oligonucleotide probes based on the gene sequences of the organism of interest. Such oligonucleotide probes have been conjugated to reporter molecules and used as molecular probes in conjunction with fluorescence and transmitted light microscopy to distinguish between different kingdoms and even different species (DeLong et al. 1989; Amann et al. 1990). Ribosomal RNA probes can also provide a means of verifying sequences obtained from organisms collected from the environment (as opposed to laboratory grown cultures). Planktonic sarcodines have resisted laboratory culture through successive generations and have consequently been difficult to study. The fact that planktonic sarcodines must be collected each time more samples were needed was one of the most challenging aspects of this thesis. Since rRNA is a very abundant in the cytoplasm of cells it provides many targets for in situ hybridizations using rRNA probes. These probes can be a valuable tool for verifying gene sequences obtained from organisms from the environment.

This thesis is organized into four chapters which are written in manuscript form. In Chapter 1, I first present the overall phylogenetic placement of the Acantharea and Polycystinea among other eukaryotes in a broad-based SSU rRNA phylogenetic analysis. In this analysis I include two representatives from the Acantharea (one Symphyacanthid representative and one Chaunacanthid representative) and four representatives of the

Spumellarida [one solitary (Thalassicolla nucleata) and three colonial spumellaria (Collosphaera globularis-huxleyi, Sphaerouzoum punctatum and Collozoum serpentinum]. In addition to phylogenetic reconstructions, data on in situ verification of the acantharian and spumellarian sequences is presented using acantharian and colonial spumellarian-specific oligonucleotide probes. This chapter also addresses the issue of the monophyly of the Actinopoda.

In Chapter 2, I present a comparison between the Thalassicolla nucleata sequence from Chapter 1 which was obtained from the Sargasso Sea with 4 additional T. nucleata sequences derived from Pacific samples. This chapter addresses variability within a single species and reexamines the known species in the genus Thalassicolla in view of the SSU rRNA sequence data obtained from specimens collected from geographically different locations.

In Chapter 3, I focus more closely on the relationships among the Spumellarida (Sphaerocollina) and more specifically, address the validity of coloniality as a legitimate phylogenetic character. In addition, to the spumellarian taxa used in Chapter 1, data from Collozoum serpentinum, Rhaphidozoum acuferum, Acrosphaera (circumtexta?) and Siphonosphaera cyathina are also utilized. Phylogenies are inferred using the same homologous positions as in the sequence alignment of Chapter 1 and using additional sites by restricting the analysis to just the colonial spumellaria.

In Chapter 4, I consider the branching patterns within the Acantharea using SSU rRNA gene sequence data from two representatives each of three orders of Acantharea, the Symphyacanthida, the Chaunacanthida and the Arthracanthida.

In Chapter 5, I summarize the conclusions of the thesis. Appendix A includes the alignment and sequence positions used in phylogenetic analyses presented in Chapters 1, 3 and 4 along with proposed locations of the secondary structure helices.

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## **Chapter 1**

# **Phylogenetic Relationships between the Acantharea and the Polycystinea (Spumellarida) Inferred from 16S-like Ribosomal RNA Gene Analyses: A Molecular Perspective on Haeckel's Radiolaria**



**ABSTRACT** The evolutionary relationship of the Acantharea and the Polycystinea (Sarcodina) to other protists was investigated using comparative small-subunit ribosomal RNA (SSU rRNA) gene analyses. While current opinion regards the Acantharea as a separate class distinct from its original Haeckelian inclusion among the "Radiolaria" ("Radiolaria" sensu lato: Polycystinea, Phaeodarea and Acantharea), most investigators continue to support the hypothesis that the Acantharea and the Polycystinea share common ancestry, as revealed by their inclusion among the superclass Actinopoda (Calkins 1909). A major impediment to using a molecular approach to ascertain whether the Actinopoda represents a formal evolutionary assemblage has been an inability to culture many of these protists in the laboratory. We collected and maintained actinopods of the classes Acantharea and Polycystinea to obtain reproductive specimens highly enriched with DNA in order to facilitate DNA extraction and sequencing. The origin of the sequences described herein were confirmed by in situ hybridization experiments. The results from molecular phylogenetic analyses inferred from SSU rRNA gene sequences do not support a shared history between the Acantharea and the Polycystinea. However, the monophyly of the Acantharea and the separate monophyly of the Polycystinea (Spumellarida) are well supported by our molecular phylogenetic analyses. The acantharian lineage branches among crown organisms while the polycystine lineage diverges prior to the radiation of the crown groups. In view of our findings, we conclude that the Actinopoda does not represent a monophyletic evolutionary assemblage and recommend that this taxonomic designation be discarded.

One morphological feature which members of the Sarcodina share is the presence of a pseudopod during some part of their life cycle. Further taxonomic division is based on the structure of these pseudopodia. Even though pseudopod-bearing protists are grouped together, there are morphological and molecular data indicating that they are polyphyletic. At the morphological level, authors have argued that a lack of morphological characters (amoeboid-form) fails to provide firm support that a group of organisms shares common ancestry (Bovee and Jahn 1973; Lee et al. 1985). At the molecular level, the polyphyly of the sarcodines has been revealed by small-subunit ribosomal DNA (SSU rDNA) analyses which place various groups of the sarcodines branching at different parts of the evolutionary tree of life (Clark and Cross 1988; Hinkle and Sogin 1993).

In the superclass Actinopoda (Calkins 1909), all members possess specialized microtubule-stiffened pseudopodia called axopodia. The taxon Actinopoda has been maintained as a phylogenetic assemblage in the most recent considerations of protistan systematics with agreement that certain heliozoa should be removed (e.g. pedinellids and heliomonads) (Cavalier-Smith 1993; Corliss 1994). The classes currently represented in this superclass include the Acantharea, Polycystinea, Phaeodarea, and Heliozoa.

One of the major distinctions between the Acantharea and the Polycystinea is the composition, architecture, and symmetry of the skeleton, when present. All acantharia form skeletons composed of monocrystals of strontium sulfate which come together at the center of the cell in a symmetrical fashion known as Müller's Law (Müller 1858). Polycystine skeletons, when present, are typically siliceous and exhibit a range of morphologies from simple spicules to more elaborate latticed shells possessing radial spines. Despite these differences, the common use of radial symmetry in cell-body plan and shell architecture often gives members of the Acantharea and the polycystine order Spumellarida a superficially similar appearance.

Although the term "radiolaria" is now often reserved as an informal taxonomic descriptor for members of the Polycystinea (Spumellarida and Nassellarida) and the Phaeodarea only, the term was originally used by biologists to include members of the class Acantharea, as well. While the term "Radiolaria" was actually coined by Johannes Müller (1858), Ernst Haeckel is credited as being the first of the early taxonomists to do an extensive description of acantharian and radiolarian systematics.

Of the 4,417 species of organisms described from collections of the Challenger Expedition, 3,508 of them were new species of Radiolaria identified by Ernst Haeckel (Haeckel 1887; Anderson 1983). In his classification scheme of the class "Radiolaria", Haeckel included four legions: the Acantharia, the Spumellaria, the Nassellaria and the Phaeodaria. This classification was later modified (Deflandre 1952; Deflandre 1953; Tregouboff 1953; Goll and Merinfeld 1979) to exclude the Acantharia (Acantharea) from the Radiolaria (the Polycystinea which included Spumellarida and Nassellarida, and the Phaeodarea). Despite some taxonomic revision, many of Haeckel's original descriptions of the Challenger Radiolaria persist today. Modern systematists, while placing acantharia in a class distinct from polycystines and phaeodaria generally agree that these classes share common ancestry and should be united within the Actinopoda (Levine et al. 1980; Lee et al. 1985; Febvre 1990; Cavalier-Smith 1993; Corliss 1994).

In all phaeodaria, polycystines and members of the acantharian order Arthracanthida, the central capsule or capsular wall divides the cell into an intracapsular region and an extracapsular region. The intracapsular region includes the nucleus, mitochondria, golgi and other major cellular machinery while the extracapsular region contains the axopodial network of the cell. Phaeodarian and polycystine radiolaria possess pores in their central capsules whereas acantharia do not.

The main features which have lead taxonomists to infer the relatedness of the Acantharea and Polycystinea are the presence of axopodia and occurrence of a central capsule, although

additional cytological similarities have been noted. Most of these similarities occur between members of the Acantharea and the Polycystinea belonging to the order Spumellarida. Most acantharia are polynucleated, but the occurrence of a single nucleus in the acantharian genus Haliommatidium has been argued as a feature it shares with the polycystines, most of which have only one nucleus. Furthermore, some authors have observed an apparent similarity between the "gelatinous pellicle" of some Sphaerellarina, a suborder in the Spumellarida, and that of some acantharia (Hollande and Enjume 1960; Massera Bottazzi 1978). Finally, the existence of strontium sulfate crystals (a skeleton-building material thought to be used only by members of the Acantharia) in some adult vegetative colonial spumellarian radiolaria and in apparently all swarmer cells of spumellaria has been suggested as potential evidence of their common ancestry (Dogel 1950; Hollande and Martoja 1974; Anderson 1981; Cavalier-Smith 1993).

Members of the Actinopoda are among the remaining protistan groups which lack any DNA sequence information with which to support or challenge the above views. We sequenced the small-subunit ribosomal RNA genes of representatives of the Acantharea and Polycystinea to determine if their assumed shared ancestry based on morphological features is supported at a molecular level. Since the taxa chosen for this study are thought to be among the most closely related of the four major classes included in the Actinopoda, this study further addresses the suitability of higher taxon-designations as Actinopoda and rekindles the debate over the best definition for "radiolaria".



## MATERIALS AND METHODS

**Sample Collection.** All specimens were collected by divers by hand using glass or polycarbonate jars. Specimens were maintained in 0.22  $\mu$ m Millipore-filtered Sargasso Sea water in glass culture tubes with brine shrimp (*Artemia salina*) as food until sacrificed for molecular analysis. All individuals were given sample designations prior to identification, and then order or genus-level classifications were made. All acantharia were collected off the southwestern coast of Bermuda in September 1994. Acantharian samples used in this paper were *Haliommatidium* sp. (BBSR 235: Order: Symphyacanthida, Family: Pseudolithidae) and Chaunacanthid 218 (BBSR 218: Order: Chaunacanthida). Polycystine radiolarian specimens, all from the order Spumellarida, were collected in a similar fashion off the southwestern coast of Bermuda on multiple dates. One solitary and three colonial spumellaria were used in this study. Solitary spumellarian *Thalassicolla nucleata* (BBS 3: Family: Thalassicollidae) was collected in May 1992, colonial spumellarian *Collosphaera globularis-huxleyi* (BBSR 173: Family: Collosphaeridae) was collected in May 1994, and colonial spumellarians *Sphaerozoum punctatum* (CR4: Family: Sphaerozoidae) and *Collozoum serpentinum* (CR16: Family: Sphaerozoidae) were collected in May 1995. Specimens used for *in situ* hybridizations were collected in September and October of 1995 in the same location.

**DNA Extraction, Amplification, Cloning and Sequencing.** In order to enrich for sarcodine DNA, whenever possible, reproductive acantharian and spumellarian radiolarian specimens were sacrificed at a point in their life cycle just prior to swarmer cell release from the central capsule. In the case of the acantharia, single individuals were collected upon formation of cysts that were generated prior to swarmer cell release. The rationale behind collecting the specimens at this point in their life cycle was twofold: first, there is a natural amplification of DNA which occurs within the organism at this time and second, many species of spumellarian radiolaria and acantharia either consume or expel

endocyttoplasmic symbiotic algae before swarmer formation, thereby reducing the potential of amplifying non-target DNA.

Individual central capsules or cysts were rinsed several times in 0.22  $\mu$ m-Millipore filtered seawater followed by a final MilliQ-water rinse prior to placement in buffer solution. *T. nucleata* specimens were processed by pooling 2 central capsules, placing them in lysis buffer (40 mM EDTA, 50 mM Tris pH 8.3, 0.75 M Sucrose) and freezing at -20°C until further processed. Cells were lysed with proteinase K (10 mg/ml) and 20% SDS and then incubated at 55°C until lysis was complete. Genomic DNA was extracted with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in TE buffer according to standard protocols.

The remaining samples were subjected to the following more streamlined protocols designed to minimize loss of DNA by minimizing the number of transfer steps (e.g. organic extractions and ethanol precipitation). Central capsules or individual cysts were rinsed as above, placed in modified 1X PCR Buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2 mM MgCl<sub>2</sub>, 0.001% Gelatin, 1.0% NP40 (Sigma)), stored frozen at -70°C and then heated at 95°C for 10 minutes to lyse cells and liberate DNA. Between 1  $\mu$ l and 5  $\mu$ l of a 20 $\mu$ l sample lysed in this manner was then used directly in PCR amplifications and typically yielded strong products.

*T. nucleata*, *C. globularis-huxleyi* and *Haliommatidium* sp. 16S-like rDNAs were amplified using PCR and eukaryotic primers specific to the ends of the molecule (Medlin et al. 1988). *T. nucleata* rDNA was cloned into M13 single strand phage, several clones were pooled and the resulting template was sequenced using Sequenase 2.0 (US Biochemical; Cleveland, OH) enzyme and existing rDNA primers (Elwood et al. 1985; Medlin et al. 1988). Two additional primers were designed and synthesized (Indiana University; Bloomington, IN) to obtain a full length sequence of *T. nucleata* (690FTnucl, 5'-AGAGGTGAAATTCAAG-3'; 690RTnucl, 5'-CTTGAATTTACCTCT-3').

Collosphaera globularis-huxleyi and Haliommatidium sp. rDNA PCR products were cloned into a double-stranded TA plasmid vector pCRII (Invitrogen; San Diego, CA) and plasmid DNA for sequencing was obtained using the Magic MiniPrep system (Promega; Madison, WI). Double-stranded sequencing of both the entire forward and reverse strands of the rDNA coding regions was accomplished using the Sequenase version 2.0 kit and methods.

**Oligonucleotide Probe Design.** In situ whole-cell hybridizations using oligonucleotide probes complementary to the sarcodine SSU rRNA were carried out in order to verify that the sequence data was derived from the sarcodine DNA and not from a contaminating source such as algal symbionts or prey material. Oligonucleotide probes were designed which were unique to the acantharian sequence data and separate probes were designed which were unique to the colonial spumellarian sequence data. At the time of design of these probes, at least single-stranded sequence data was available representing three orders of Acantharea; Arthracanthida, Chaunacanthida and Symphyacanthida (Haliommatidium sp.), with which to search for signature sequences for designing probes (Chapter 4). For the colonial spumellarian radiolaria, at least single-stranded sequence information from C. globularis-huxleyi and Rhaphidozoum acuferum (see Chapter 3) was available.

Probes were designed which would target either the Acantharea or the colonial spumellaria (Fig. 1). Only colonial spumellarian probes were designed because it was not possible to find signature sequences which were sufficiently unique (having at least three base pair mismatches against any other SSU sequences in the RDP database) to design probes which would identify both the solitary radiolarian T. nucleata and the two colonials. The biotin-labeled probes designed for acantharian samples were as follows: A497bio, 5'-TCATTCCAATCAACTCAC-3'; A899bio, 5'-TCGTCATACAAAGGTCCA-3'. The probes designed for colonial spumellarian samples were as follows: R906bio, 5'-AAC-



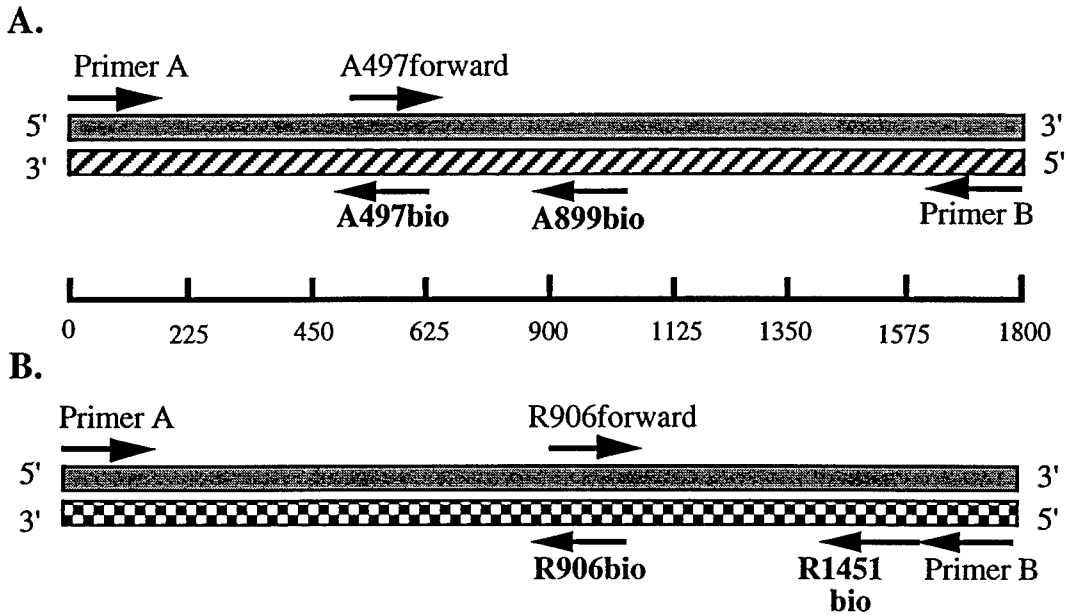


FIG. 1. A. The locations of acantharian-specific oligonucleotide probes (in bold) A497bio and A899bio used in *in situ* hybridization experiments of acantharia. The complement of A497bio was synthesized (denoted as A497forward in the schematic) and used in combination with Primer B in PCR reactions to later specifically amplify the acantharian gene fragment approximately 1,300 bp in length. Primer A was then used in combination with A899bio to obtain an overlapping fragment to the previous one approximately 900 bp in length to obtain the rest of the gene. B. The locations of colonial spumellarian-specific oligonucleotide probes (in bold) R906bio and R1451bio used in *in situ* hybridization experiments of colonial spumellaria. The complement of R906bio was synthesized (denoted as R906forward in the schematic) and used in combination with Primer B in PCR reactions to specifically amplify the gene fragment approximately 900 bp in length. Primer A was used in combination with R1451bio to obtain an overlapping fragment to the previous one approximately 1,450 bp in length to obtain the rest of the gene.

GATAAAATACTAATA-3'; R1451bio, 5'-TATTGTAGCCCGTGCGCT-3'. All probes were analyzed by Check Probe (RDP; University of Illinois, Urbana) for potential homology to other SSU rRNA sequences in the database before syntheses were carried out. The oligonucleotide probes A899bio and R1451bio were synthesized by Eppendorf (Madison, WI) and oligonucleotide probes A497bio and R906bio were synthesized by Cruachem (Foster City, CA). The following eukaryote-specific, 5'-biotinylated probes were used as positive controls: EUK502Rbio; 5'-ACCAGACTTGCCCTCC-3' (Amann et al. 1990) and EUK1209Rbio; 5'-GGGCATCACAGACCTG-3' (Giovannoni et al. 1988). These probes will hybridize with all known eukaryotic SSU rRNA.

**In situ Hybridizations.** In situ hybridizations were carried out using both fluorescence and colorimetric detection methods. The latter technique was necessary for the colonial spumellarian samples due to severe autofluorescence occurring in these organisms. Fluorescence in situ hybridizations on acantharia were carried out as described in Lim et al. (1993) using biotinylated probes and detection with FITC-avidin solution (20 µg/ml in 100 mM NaHCO<sub>3</sub>-buffered saline, pH 8.2; (Vector Laboratories, Inc.; Burlingame, CA)). Acantharia were fixed for 1 hour at 4°C in 1X Histochoice (Amresco; Solon, OH) fixative diluted in 0.22 µm-filtered Sargasso seawater. Individuals were then transferred to gel-subbed-slides, overlaid with 0.05% agarose and allowed to dry overnight. Probe was added to a final concentration of 5 ng/µl. Probe treatments consisted of a negative control (incubation in fluorescein-labeled avidin with no probe added), a positive control (biotinylated EUK 1209Rbio added), and two separate acantharian-specific probe treatments using A497bio and A899bio oligonucleotides respectively.

Hybridizations were carried out at 42°C for 6-8 hours and subsequent washes were done at 45°C. Cells were mounted in Citifluor immersion oil (Citifluor, Ltd.; London, England) and viewed on a Zeiss Axiophot equipped for epifluorescence microscopy.

Epifluorescence photomicrographs were taken with an integral camera system using a

fluorescein isothiocyanate (FITC) filter set combination consisting of a 450-490 nm band-pass excitation filter; a 510 nm long-pass dichroic mirror; and a 515-565 nm band-pass emission filter. Fuji 100 ASA Provia color slide film was used for fluorescence pictures. All exposure times for a set of samples (i. e. negative control, positive control, taxon-specific probes) were kept constant so that the relative intensity was indicative of probe binding. Transmitted light photomicrographs were also taken of the same specimens using Kodak ASA 160 Tungsten film.

Colorimetric-based in situ hybridizations were carried out on colonial spumellarian samples using the Gibco BRL In Situ Hybridization and Detection System (Life Technologies; Frederick, MD) with the following modifications for use with rRNA and larger sarcodines. Colonies were preserved in 1X Histochoice with 95% ethanol added in a ratio of 4:1. Colonies were preserved for 1 hour at 4°C, transferred to 70% ethanol and held overnight at 4°C. Aliquots of preserved central capsules from a single colony were placed on silanated glass slides (Midwest Scientific; St. Louis, MO) and allowed to air dry. Slides were then baked at 65°C for 1 hour to remove endogenous alkaline phosphatase activity. Hybridizations were carried out in 50 µl-capacity Probe-Clip "Press-to-Seal" incubation chambers and holders (Midwest Scientific; St. Louis, MO). Four probe treatments were carried out using central capsules from the same colony: a negative control incubation (streptavidin-alkaline phosphatase conjugate with no probe added), a negative probe control (A899bio acantharian probe added), a positive probe control (EUK 502bio and EUK 1209bio added), and a colonial spumellarian probe treatment (R906bio and R1451bio added). All probe treatments contained final total probe concentrations of 1 ng/µl.

Hybridizations were conducted according to the manufacturer's instructions for "DNA Detection" with the above modifications and the omission of any steps specifically required for DNA targets. Slides were hybridized for 8 hours and probe detection was carried out

according to manufacturer's protocol with levamisole (Sigma; St. Louis, MO) added at 200 µg/ml upon addition of alkaline phosphatase conjugate to further eliminate any potential endogenous alkaline phosphatase activity. Developed slides were permanently mounted in Crystal/Mount (Biomedex; Foster City, CA) and observed on a Zeiss standard microscope equipped with phase microscopy. Transmitted light photomicrographs of samples were taken with an Olympus OM4-T camera using Kodak 160 speed Tungsten film.

**Direct Sequencing of PCR Products.** Upon achieving successful in situ hybridizations, further amplifications were accomplished using group-specific probes as primers in PCR reactions to specifically amplify and sequence sarcodine rDNA. The acantharian probe A899bio was used as a reverse primer in combination with Medlin amplification-primer A (Medlin et al. 1988) to specifically amplify the first 900 base pairs of acantharian SSU rRNA genes from the chaunacanthid sample BBSR 218 (See Fig. 1). The complement of probe A497 (non-biotinylated) was synthesized (Cruachem) and used in combination with Medlin amplification primer B (Medlin et al. 1988) to amplify a gene fragment approximately 1,300 base pairs in length which overlapped the primer A/A899bio amplification fragment.

Likewise for the colonial spumellaria, the complement of probe R906bio was synthesized (Cruachem) and the primer A/R1451bio and R906/primer B primer-pair amplifications were carried out on colonial spumellarian samples CR4 and CR16 (See Fig. 1). All PCR fragments were purified using the Wizard PCR Prep system (Promega; Madison, WI). Direct sequencing of PCR products was accomplished using reagents from the Sequitherm Long Read Sequencing Kit (Epicentre Technologies; Madison, WI) along with the Sequitherm Cycle sequencing protocol developed by Li-Cor which consisted of 5 minutes of denaturation at 95°C prior to 30 cycles of 20 sec at 95°C, 30 sec at 60°C, and 1 minute at 70°C using a Perkin Elmer 2400 Thermo Cycler. Sequenced templates were run out on a Licor model 4000L sequencer. Gel images were transferred from Licor to BioImage



(Millipore Corp.; Ann Arbor, MI) and sequences were analyzed using the Millipore BioImage DNA Sequence Film Reader software.

**Phylogenetic Analysis.** The 16S-like rRNA sequences of acantharian and radiolarian samples were aligned against a subset of the total eukaryotic alignment data base (Olsen et al. 1992). The 31 taxa included in this study are listed in Table 1. Sequences were aligned by eye using the Olsen Multiple Sequence Alignment Editing program with regard to primary and secondary structural conservation. 1,369 positions were used in the phylogenetic analyses. A distance matrix based on pairwise distances was created for the data set and a phylogenetic tree was inferred from these data by the method of Olsen (Olsen 1988). One hundred bootstrap replicates were conducted and a consensus tree was obtained using PHYLIP 3.5 (Felsenstein 1985). Phylogenetic trees were also inferred by the maximum likelihood method in conjunction with the fastDNAm1 program (Olsen et al. 1994) using a generalized two parameter model of evolution (Kishino and Hasegawa 1989) and maximum parsimony method using PAUP, version 3.1.1 (Swofford 1991). The maximum parsimony tree was obtained from a consensus of 100 bootstrap replications which were conducted using a heuristic search option with random addition sequence, 10 replicates and the tree bisection-reconnection algorithm. Identical phylogenetic analyses as those described above were also performed with Phreatamoeba balamuthi removed from the data set, in order to determine stability of the relative branching of the acantharia and the polycystine radiolaria. In these analyses, the same alignment and sequence positions were used as in those analyses including Phreatamoeba balamuthi in the data set.

## RESULTS

In situ hybridization experiments confirmed the origin of the acantharian and spumellarian sequences (Fig. 2). Acantharian specific probes were found to specifically hybridize to the acantharia (Fig. 2, panels F and H) and not to colonial spumellaria (Fig. 2, panel J).

Table 1. Percent G + C content and taxonomic affinities of the taxa used in this study.

| Species                          | SSU rDNA |                    |
|----------------------------------|----------|--------------------|
|                                  | G+C (%)  | Taxonomic affinity |
| <u>Theileria annulata</u>        | 45       | Apicomplexa        |
| <u>Symbiodinium pilosum</u>      | 45       | Dinoflagellida     |
| <u>Oxytricha granulifera</u>     | 46       | Ciliophora         |
| <u>Blepharisma americanum</u>    | 47       | Ciliophora         |
| <u>Porphyridium aerugineum</u>   | 48       | Rhodophyta         |
| <u>Stylonema alsidii</u>         | 46       | Rhodophyta         |
| <u>Emiliana huxleyi</u>          | 50       | Haptophyta         |
| <u>Labyrinthuloides minuta</u>   | 44       | Labyrinthulid      |
| <u>Ochromonas danica</u>         | 45       | Chrysophyceae      |
| <u>Cafeteria roenbergensis</u>   | 47       | Bicosoecids        |
| <u>Chlamydomonas reinhardtii</u> | 50       | Chlorophyte        |
| <u>Oryza sativa</u>              | 51       | Plantae            |
| <u>Acanthamoeba castellanii</u>  | 52       | Amoebida           |
| <u>Hartmanella vermiformis</u>   | 49       | Amoebida           |
| <u>Athelia bombacina</u>         | 47       | Fungi (Eumycota)   |
| <u>Blastocladiella emersonii</u> | 46       | Fungi (Eumycota)   |
| <u>Mnemiopsis leidyi</u>         | 47       | Animalia           |
| <u>Diaphanoeca grandis</u>       | 44       | Choanoflagellate   |
| <u>Phreatamoeba balamuthi</u>    | 47       | Amoeba             |

Table 1. (cont.)

| Species                                | SSU rDNA |                           |
|--|----------|---------------------------|
|  | G+C (%)  | Taxonomic affinity        |
| <u>Paulinella chromatophora</u>        | 48       | Filosea                   |
| <u>Euglypha rotunda</u>                | 45       | Filosea                   |
| <u>Haliommatidium</u> sp.              | 44       | Symphyacanthida           |
| Chaunacanthid 218                      | 45       | Chaunacanthida            |
| <u>Dictyostelium discoideum</u>        | 42       | Dictyostelida             |
| <u>Physarum polycephalum</u>           | 52       | Plasmodial Slime<br>Molds |
| <u>Thalassicolla nucleata</u>          | 36       | Spumellarida              |
| <u>Collosphaera globularis-huxleyi</u> | 35       | Spumellarida              |
| <u>Sphaerozoum punctatum</u>           | 37       | Spumellarida              |
| <u>Collozoum serpentinum</u>           | 38       | Spumellarida              |
| <u>Entamoeba gingivalis</u>            | 34       | Amoebida                  |
| <u>Naegleria gruberi</u>               | 48       | Schizopyrenida            |

Likewise, colonial spumellarian probes specifically hybridized with colonial spumellaria (Fig. 2, panel L).

The % G + C content of the SSU rRNA gene for Haliommatidum sp. and Chaunacanthid 218 were 44% and 45% respectively, which was similar to many of the other taxa used in the analyses (Table 1). However, spumellarian % G+ C content values (35% - 38%) were similar to that of Entamoeba gingivalis (34%) and were low relative to typical eukaryotic values which are usually around 50%. Gene lengths in base pairs (bp) for acantharian and spumellarian samples were typical for eukaryotic SSU rRNA genes. Haliommatidum sp. and Chaunacanthid 218 were 1788 bp and 1778 bp. Lengths of genes for spumellaria were as follows: T. nucleata, 1770 bp; C. globularis-huxleyi, 1797 bp ; S. punctatum, 1788 bp; C. serpentinum, 1798 bp.

The phylogenetic trees inferred by the distance-matrix, maximum parsimony (Fig. 3) and maximum likelihood (data not shown) methods clearly rejected a common ancestry between these two groups of actinopods. Numbers at nodes represent bootstrap values as a percentage of 100 resamplings of the data set. Only bootstrap values greater than 50% are shown and represent relative measures of confidence. Both the distance and parsimony trees placed the spumellarian radiolaria branching as a diverging lineage below the "crown" groups (Knoll 1992), those taxa representing major eukaryotic assemblages simultaneously radiating from the node labeled with a bootstrap value of 85/65. Both methods revealed a poorly resolved branching point for the acantharia (Haliommatidum sp. and Chaunacanthid 218) among the crown radiation. The relative positions of the acantharia and the polycystine radiolaria were not affected by removal of Phreatamoeba balamuthi (Fig. 4). Removal of Phreatamoeba balamuthi from the data set resulted in higher bootstrap support values for the node leading to the crown (89/98).

A low bootstrap support value of 67% was obtained for the branching of the spumellaria with Entamoeba gingivalis in the parsimony analysis, but this support was not observed in



FIG. 2. In situ hybridization of Histochoice-preserved specimens using oligodeoxynucleotide probes complementary to the 16S-like (small-subunit) ribosomal RNA sequences of acantharia (A-H) and colonial spumellaria (I-L). For both acantharian and colonial spumellarian cells, probes conjugated to biotin were detected by either fluoroisothiocyanate (FITC)-avidin or streptavidin-alkaline phosphatase-conjugated secondary labels. For the acantharian cells, hybridization detection was carried out using epifluorescence microscopy with settings specific for FITC excitation (panels B, D, F, H). Colonial spumellarian cells were viewed using phase contrast microscopy and hybridizations were detected colorimetrically using the localized, purple precipitate of the enzymatic reaction of alkaline phosphatase on nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) substrates. Panels A-H depict four different acantharian cells of the same species with corresponding phase and epifluorescence photomicrographs of the same cell. Scale bars represent 75  $\mu$ m in panels A-H. Panels A and B show the negative control to which only FITC-avidin was added. Note the minimal background fluorescence of the cell under epifluorescence (B). Panels C and D show the positive control treatment to which a eukaryotic-specific probe designed to target all eukaryotes (EUK 1209R) was added. Panels E and F and G and H show the probing of cells with two different acantharian probes (F, A497; H, A899), both designed against members of three different orders of acantharia (i.e. these probes should hybridize with all species within these orders of acantharia). Panels I through L show hybridization results for single individuals within the same colony. Panel I shows the negative control to which only the streptavidin-alkaline phosphatase-conjugated secondary label was added. Panel J shows a negative probe control treatment to which an acantharian probe was added. Panel K shows the results of the positive control hybridization with eukaryote probes (EUK502R and EUK1209R) and panel L shows hybridization with colonial spumellarian probes (R906 and R1451). Scale bars represent 35  $\mu$ m in panels I through L.

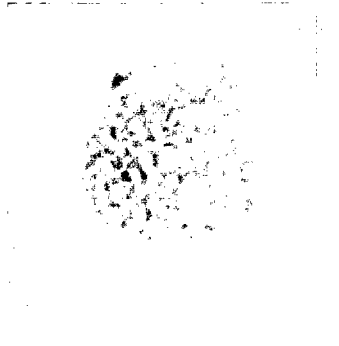
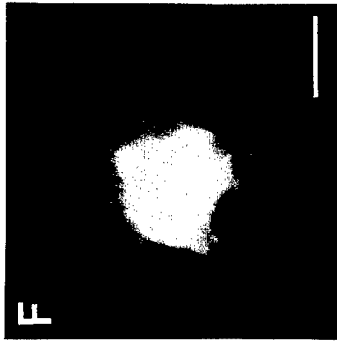
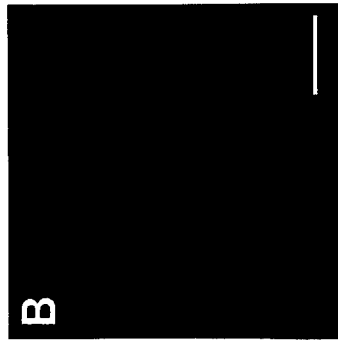
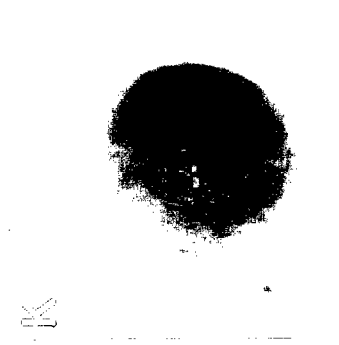
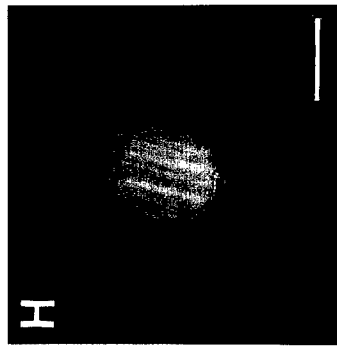
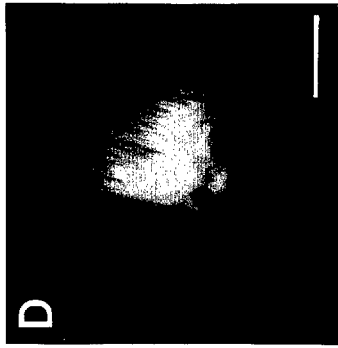
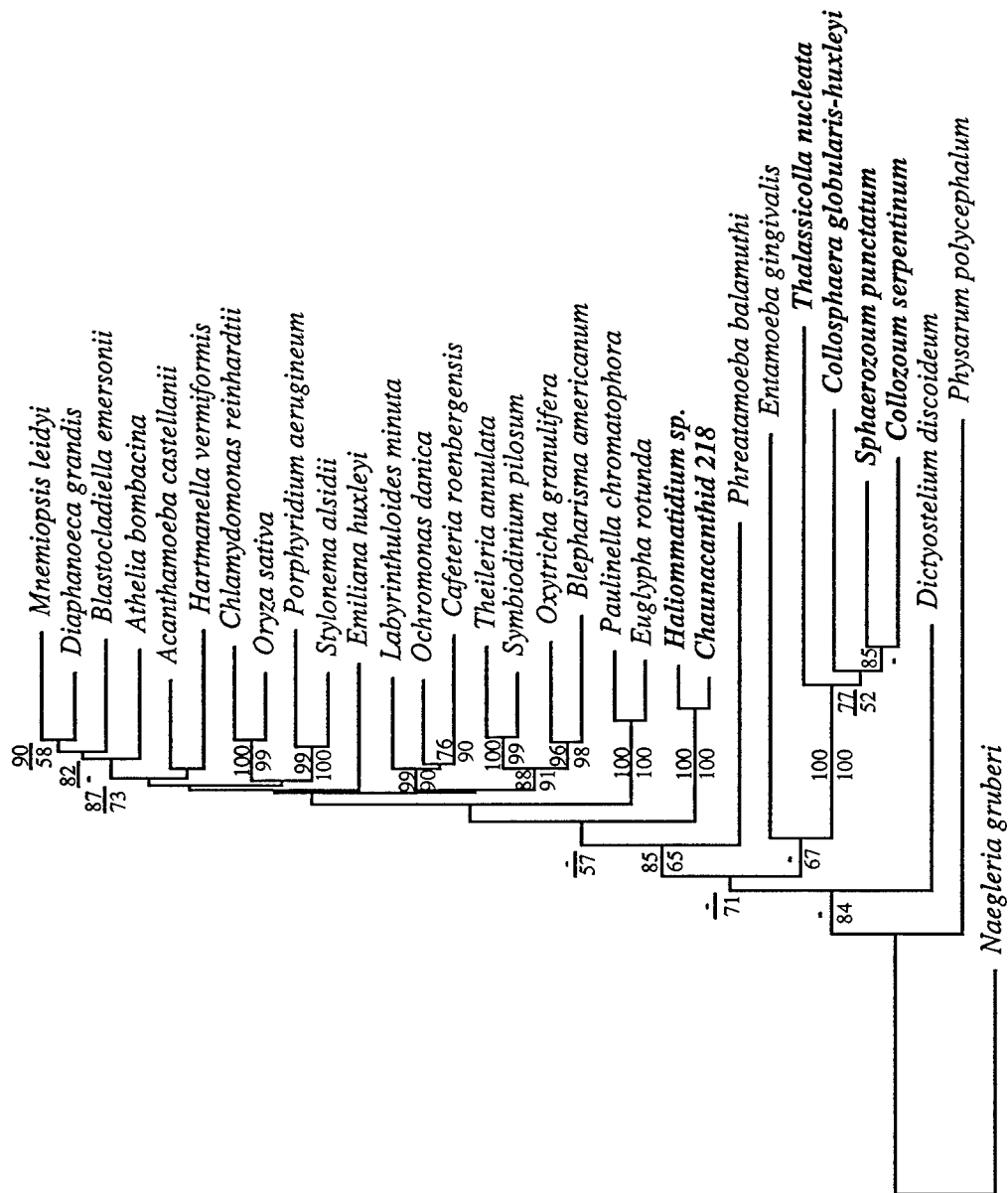






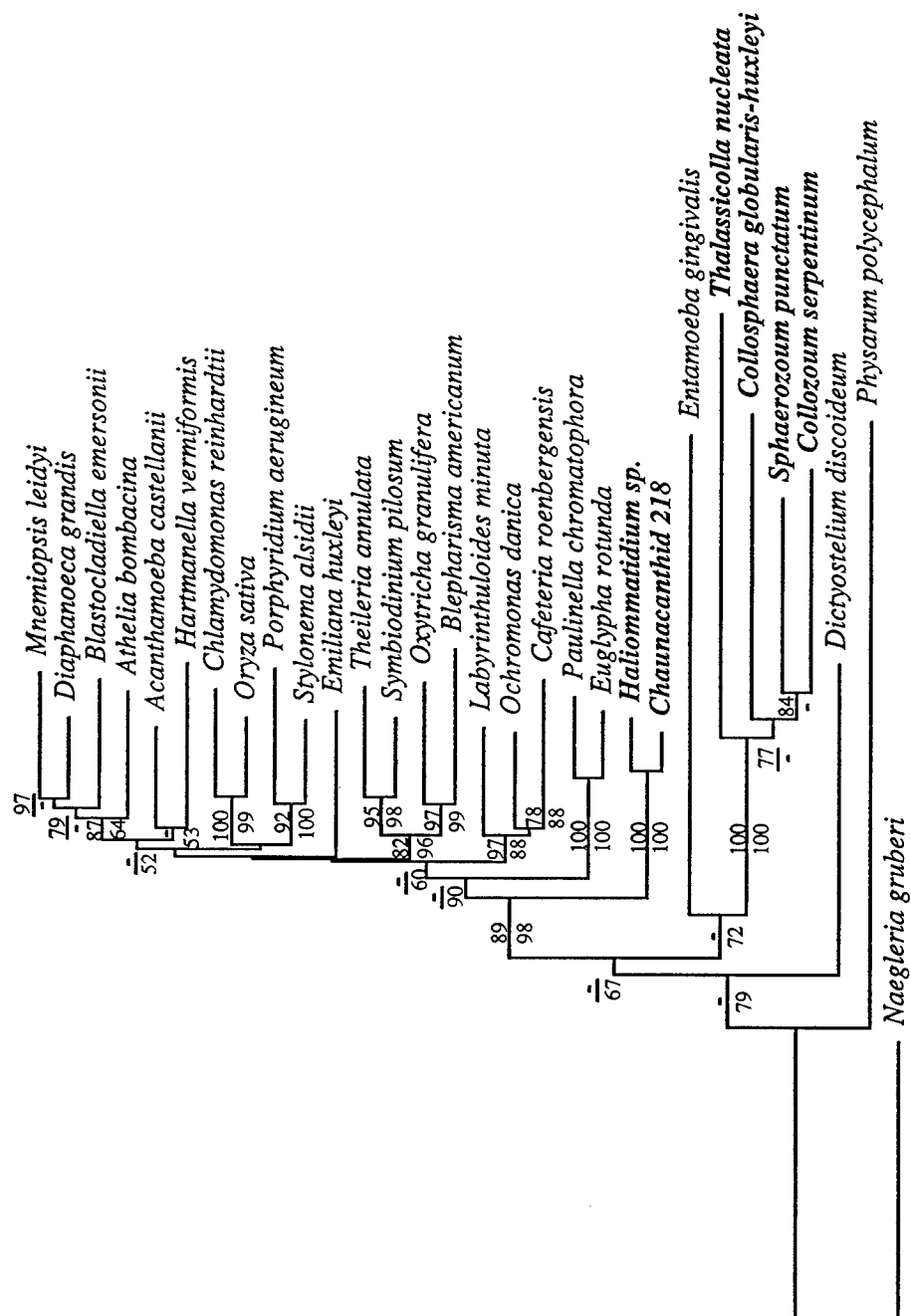


FIG. 3. The inferred phylogeny for the acantharia and the spumellarian radiolaria. A distance tree is shown. Numbers at the nodes represent bootstrap values, given as a percentage of 100 resamplings of the data. Bootstrap values for distance analyses are given above the line, whereas maximum parsimony values are below. A dash indicates that the bootstrap value for that node was below 50% in the method used for phylogeny reconstruction. The bar insert corresponds to 10 changes per 100 nucleotide positions. Only horizontal components of the tree are measures of evolutionary distance.



10 changes per 100 nucleotides

FIG. 4. The inferred phylogeny for the acantharia and the spumellarian radiolaria after the removal of Phreatamoeba balamuthi from the data set. A distance tree is shown. Numbers at the nodes represent bootstrap values, given as a percentage of 100 resamplings of the data. Bootstrap values for distance analyses are given above the line, whereas maximum parsimony values are below. A dash indicates that the bootstrap value for that node was below 50% in the method used for phylogeny reconstruction. The bar insert corresponds to 10 changes per 100 nucleotide positions. Only horizontal components of the tree are measures of evolutionary distance.



10 changes per 100 nucleotides

the distance analysis nor the topology of the maximum likelihood analysis (data not shown). No other potential immediate common ancestors were indicated by these data. The position of the spumellaria relative to other groups branching below the crown varied between the distance and parsimony analyses. Therefore, the exact branching order of the spumellarian radiolaria also remains unresolved at this time. The monophyly of the acantharia and the monophyly of the spumellaria, however, were well supported (100% in all cases).

The branching patterns within the spumellaria in the distance and the parsimony analyses both showed the solitary spumellarian T. nucleata branching prior to the colonial spumellaria. Although the bootstrap support for this node was barely above 50% in the parsimony analysis, a higher bootstrap value was obtained (77%) with distance methods. The relationship between the solitary and colonial spumellaria has been examined using a larger suite of spumellarian taxa and additional nucleotide sites in Chapter 3.

## DISCUSSION

The relative positions of the acantharia and the spumellaria in molecular phylogenetic trees indicate that the presence of axopodia, a capsule membrane and the ability to metabolize strontium sulfate should be reconsidered as reliable phylogenetic markers. Our molecular study of acantharian and spumellarian phylogeny strongly agrees with what has been speculated in the literature over the past several years: axopodia have evolved more than once and most likely represent convergent structures created in response to similar ecological constraints through evolutionary time (Merinfeld 1978; Shulman and Reshetnyak 1980; Merinfeld 1981; Reshetnyak 1981a). An independent evolution of axopodia within the chromistan Pedinellea of the Heliozoa already has been suggested on morphological grounds (Cavalier-Smith 1993). Given the results of this study, retention of the superclass Actinopoda seems inappropriate, as does the adoption of the new phylum Radiozoa, which

has been described as a modern-day Radiolaria sensu lato (Cavalier-Smith 1993; Corliss 1994).

The presence of a central capsule and the ability to secrete strontium sulfate have been described as two synapomorphies defining the Radiozoa (Cavalier-Smith 1993). However, the central capsule found in spumellaria and that which exists in one order of Acantharea (the Arthracanthida) have been shown to differ (Massera Bottazzi 1978; Reshetnyak 1981a). The acantharian central capsule in this order is non-perforated and of ectoplasmic origin while that of the polycystines and the phaeodaria is perforated and located between the ectoplasm and the endoplasm. Furthermore, the presence of central capsules in the Arthracanthida, which are considered to be more derived than other orders of acantharia which lack central capsules (Hollande et al. 1965; Strelkov and Reshetnyak 1974; Reshetnyak 1981a), suggests that "central capsules" may have evolved more than once.

The occurrence of strontium sulfate in both acantharia and spumellaria is another feature often cited as evidence of their common ancestry. Vegetative adults of colonial spumellaria are known to house crystals of strontium sulfate in their central capsules and the biflagellated swimmers of all spumellarian radiolaria examined thus far contain crystals of strontium sulfate in membrane bound vesicles. However, metabolism of strontium sulfate is not unique to the acantharia and spumellarian radiolaria. Crystals of strontium sulfate have been observed in the desmid alga Closterium littorale (Raven et al. 1986), in Chara, the "stonewort" freshwater plant, and in loxodid ciliates (Fenchel and Finlay 1986). The role of strontium sulfate in Chara and the loxodid ciliates is apparently graviperception (Fenchel and Finlay 1986; Raven et al. 1986). A similar function has been proposed in the desmid algae (Raven et al. 1986). This function apparently has never been proposed for the membrane-bound crystals found in spumellarian swimmers. Instead, Anderson (1981) has suggested that strontium sulfate crystals may serve a function in buoyancy control but

admits that silica or calcium compounds, which occur at higher concentrations in sea water, would be better candidates for this purpose. Anderson also suggested that strontium may be of some physiological importance to the spumellaria but does not elaborate on what this requirement might be. One possibility is that strontium serves a similar function in spumellaria as in some gastropods where it is required for proper shell development (Bidwell et al. 1986). A caveat to the potential importance of strontium in spumellarian skeletal development, however, is that even spumellarian species which lack skeletal material, like T. nucleata and Collozoum spp., have swarmer with crystalline strontium sulfate inclusions. Furthermore, the lack of strontium sulfate crystals in acantharian swarmer cells, seems inconsistent with the idea that strontium sulfate serves a similar function in both the Acantharea and the spumellarian polycystines.

While most of the literature has favored a common ancestry of the acantharia and the spumellaria, a series of papers published in Russian during the early 1980's argued against this idea (Shulman and Reshetnyak 1980; Reshetnyak 1981a; Reshetnyak 1981b). These papers describe several morphological features as unique to the Acantharea. These major features include the existence of a skeleton of strontium sulfate, not merely crystals of the compound as are found in some spumellaria, organized in a highly geometrical fashion according to Müller's Law. Also thought to be unique to Acantharea is the cytoplasmic feature called the calymma which, along with the ectoplasmic cortex and the non-actin containing myonemes, forms a "hydrostatic apparatus" thought to render acantharia capable of movement in the vertical direction. These authors concluded that the axopodial system was not a reliable phylogenetic marker, and defended their argument by comparison of ultrastructural studies of the axopodial systems in different groups of Actinopoda (Hollande 1953; Cachon and Cachon 1964; Febvre 1971; Cachon and Cachon 1972; Febvre 1972; Cachon et al. 1973; Febvre 1973).



In brief, the Russian authors proposed that axopodial systems evolved independently several times in evolution as amoeboid-like protists were going from benthic to pelagic modes of existence. The authors pointed out differences in the axopodial systems of various classes within the Actinopoda in support of their interpretation of the ultrastructural data provided by the French investigators cited above. They pointed out structural differences in the axoneme (the microtubular shaft which stiffens axopodia) and differences in the size and location of the axoplast (the microtubule-organizing center of the axoneme) between taxa which they say is suggestive of convergence not homology. Finally, they mention the presumed artificial grouping of actinophrid and centrohelid heliozoa (which possess very different axoneme structures) in support for their argument. For more details and diagrams comparing actinopod axopodial systems the reader is referred to the Russian literature cited above for which fairly complete translations are available from LAZ. The results from the molecular work described in this thesis support the major claims made by these Russian authors.

The absence of strontium sulfate in swarmer cells of Acantharea, as mentioned above, is noteworthy in this discussion because it suggests yet another difference between the respective requirements of acantharian and spumellarian swimmers. Given the fact that strontium sulfate crystals are thought to be involved in buoyancy control, their absence in acantharian swimmers, and the fact the acantharia are understood to reproduce at depth (Reshetnyak 1981a), the following scenario is consistent with what is currently understood about acantharian biology: Perhaps the need for strontium sulfate crystals for buoyancy control in acantharian swimmers is overcome by the ability of acantharia to regulate their depth in the water column via their "hydrostatic apparatus", allowing the acantharian to sink to the desired depth for release of its swarmer cells. In addition, many species of acantharia form cysts also composed of strontium sulfate which aid in the sinking of swarmer cells to depth. The greater density of strontium sulfate relative to silicon dioxide

may also explain why polycystine radiolaria, which can possess siliceous skeletons, utilize the heavier strontium sulfate in their swarmers. Interestingly, phaeodarian radiolaria which live deeper in the water column do not have the capacity to metabolize strontium sulfate and, like acantharia, lack strontium sulfate-containing swarmer cells. Whatever function served, the presence of strontium sulfate in these marine protists and its singular utilization as the structural compound in the skeletons of acantharia deserves further scrutiny in the evolution of this group as do their unique non-actin-containing myonemes.

Which protists, then, share most recent ancestry with the Acantharea? The branching pattern of the acantharia was strikingly shallow relative to that of the spumellarian radiolaria, possibly suggesting that the acantharia diversified more recently than the spumellaria. The most recent common ancestor of the Acantharea could possibly be found among actinopods which have been placed among the incertae sedis. Among them we find such specimens as Podactinelius sessilis (Schröder 1907), possibly the only living benthic acantharian, which was described aboard the Deutschen Südpolar-Expedition of 1901-1903. This genus was once included as a separate order Actineliida in the class Acantharea. However, since the last publication of the Committee on Systematics and Evolution of the Society of Protozoologists, it been relegated to an uncertain taxonomic affinity. This genus possesses spines of strontium sulfate (400-500) which are not arranged in the characteristic geometric pattern observed in all Acantharea.

The determination of the nearest relative of the Spumellarida remains equally challenging. If the long branches occurring in the spumellarian lineage may be interpreted as evidence of their ancient origins it may be difficult to determine the phenotype of the most recent common ancestor of the Spumellarida. Although the fossil record of spicule-bearing forms (Sphaerzoidae) extends to the Lower Oligocene (Bjørklund and Goll 1979) and that of the Collosphaeridae to the base of the Miocene (Riedel 1967), even more ancient origins are possible in view of the existence of extant skeletonless forms which would not be

preserved in the sediments. As an alternative hypotheses, the long branch lengths of the spumellaria may be explained as the result of a rapidly evolving lineage.

It is assumed that the Nassellarida which represent the second order included in the Polycystinea are closely related to the Spumellarida (Cachon et al. 1990). The molecular phylogenetic position of the Phaeodarea is also unknown and deserves consideration. As for Haeckel's Radiolaria and the definition of the Radiolaria sensu lato (Polycystinea, Phaeodarea and Acantharea), continued use of this definition in anything but a historical perspective, and the biological implications behind it appear unjustified in view of the results described herein.

## ACKNOWLEDGMENTS

This material is based upon work supported under a National Science Foundation Minority Graduate Fellowship. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation. Further financial support is acknowledged in part from NSF (OCE-9314533) (to D. A. Caron) and from the Scurlock Fund for research at the Bermuda Biological Station for Research, Inc. (BBSR), the Grant-In-Aid Fund of BBSR, funds from the Woods Hole Coastal Research Center, the Education Office of the Woods Hole Oceanographic Institution and the Lerner Gray Fund for Marine Research of the American Museum of Natural History (to L. A. Amaral Zettler). We thank Drs. Annika Sanfilippo and William Riedel for offering reprints of their translations of Russian references in their collection, as well as, making available other hard-to-find references. We thank Dr. Rebecca Gast for critical reading of this manuscript. We thank Dr. Alexi Shalapyonok for assistance with translating Russian manuscripts and Gaspar Taroncher for assistance in German translation. We wish to thank Dr. Anthony Michaels and Dr. O. Roger Anderson for help with identifications of specimens. We also wish to further

acknowledge the assistance of Dr. Anthony Michaels for opening up his lab at the Bermuda Biological Station for Research, Inc. (BBSR) and allowing lab members especially Frances Howes and numerous other volunteers to assist with blue water diving collection of specimens. We also wish to express thanks to Dr. Hank Trapido-Rosenthal for use of his lab and equipment at BBSR for in situ hybridization work.

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## **Chapter 2**

### **Insights on the Diversity within a "species" of Thalassicolla (Spumellarida) Based on Small-Subunit Ribosomal RNA Gene Sequencing**



**ABSTRACT.** We compared small-subunit ribosomal RNA gene sequences of samples from solitary spumellarian radiolarian Thalassicolla nucleata collected from the Sargasso Sea and the Pacific Ocean. Sequences derived from these separate locations showed variability in both length and base-pair composition which is consistent with genus-level variation reported in the literature for other taxa. The seven existing descriptions of Thalassicolla species, including T. nucleata, are discussed in view of these molecular findings and with reference to our current understanding of the physiology and life cycle of the spumellarian radiolaria.

**Supplementary key words.** Actinopoda, Polycystinea, radiolarian, sarcodine

Little systematic revision has occurred in the genus Thalassicolla since its first representative, Thalassicolla nucleata was described by Thomas Huxley in 1851. The solitary spumellarian T. nucleata along with many colonial spumellaria, all to which Huxley assigned the name Thalassicolla punctata, were among the first described living polycystine radiolaria. Thalassicolla punctata was later dissolved by Johannes Müller, but T. nucleata was retained and is still recognized as a valid species today.

The six other species of the genus Thalassicolla were all proposed by Ernst Haeckel primarily from specimens collected aboard the H. M. S. Challenger (Haeckel 1887). These species included the following: T. pellucida, T. spumida, T. zanclea, T. australis, T. maculata and T. melacapsa. Haeckel used qualities of the central capsule such as wall texture, color and size as the major distinguishing features upon which to separate species of Thalassicolla. Curiously, of the seven known species of Thalassicolla, only four, T. pellucida, T. spumida, T. melacapsa and T. nucleata appear to be mentioned in the literature since Haeckel's first reports, and no systematic revisions of the species of Thalassicolla have occurred since Haeckel's time.

As more information about the physiology and life cycle of this genus has been obtained, the validity of some of Haeckel's species descriptions have been questioned. Most of these studies have been carried out on the single species T. nucleata.

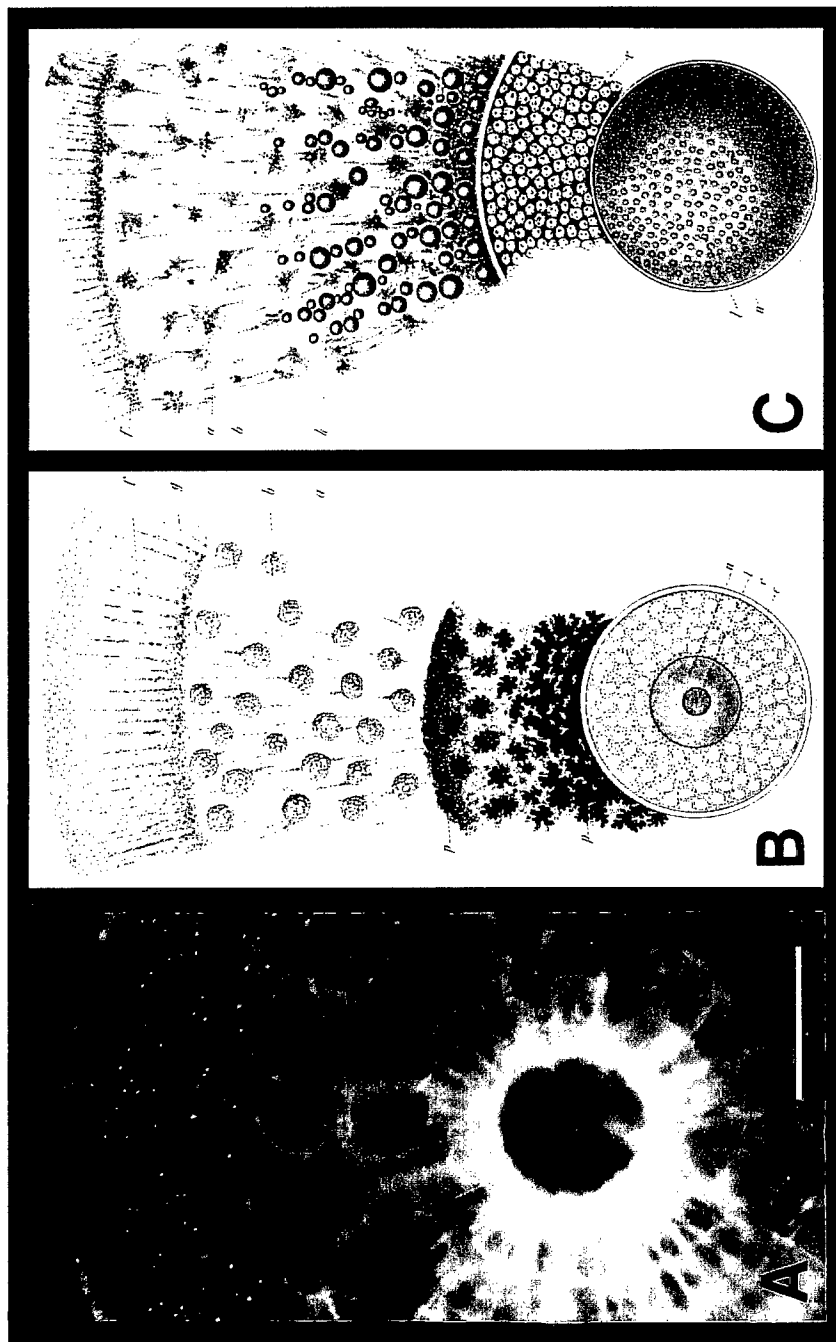
Since its original description, T. nucleata has become a model organism for research into the biology of the spumellarian radiolaria (Anderson 1978; Herring 1979; Anderson 1983). Thalassicolla nucleata is a large (3 - 5 mm) solitary, spumellarian polycystine found ubiquitously in tropical and subtropical surface waters of open-ocean communities including the Sargasso Sea and the Pacific Ocean, as well as other locations. Thalassicolla nucleata lacks a skeleton and represents one of the simplest examples of polycystine cell architecture: a single central capsule, usually enveloped by a dark opaque layer, which is in

turn surrounded by a matrix of highly-alveolated, symbiont filled extracapsular material (see Fig. 1A).

Given the rather simple cell-architecture of T. nucleata, it seems possible that the criteria used by Haeckel to distinguish between different species of Thalassicolla may not have been reflected on the genetic level. The morphological differences noted by Haeckel may have been the result of the physiological state of the cell, life cycle effects, or environmental influences. Ultimately it may not be possible to distinguish between species of Thalassicolla based on morphological criteria alone.

We approached the question of species diversity in the genus Thalassicolla by comparing gene sequences of the small subunit ribosomal RNA (SSU rRNA) in T. nucleata collected from different locations to determine if there are genetic differences which are not associated with morphological details at the species level. Two features of the life history make T. nucleata well-suited for molecular phylogenetic study. First, although it cannot be reared through successive generations, T. nucleata readily undergoes swarmer formation in the laboratory during which time the dark opaque layer surrounding the central capsular region of the cell is shed revealing a milky-white capsule beneath it (Anderson 1978). Swarmer formation marks the onset of reproduction in the cell, at which time the intracapsular DNA concentration is significantly increased and divided among swarmer cells. This "natural" amplification of DNA within the cell greatly facilitates retrieval of DNA for molecular analysis. Second, like many spumellarian radiolaria, T. nucleata lives in association with symbiotic algae which are believed to enhance survival of species in oligotrophic environments (Anderson 1978; Anderson and Botfield 1983). These algae might normally complicate separation of host DNA from symbiont DNA, but the symbionts in polycystines are physically excluded from the central capsular region by the capsular membrane. Dissection of the central capsule away from the rest of the extracapsular material which houses symbionts, along with sacrificing the cell immediately prior to

Fig. 1. A. A transmitted-light photomicrograph of a typical specimen of Thalassicoilla nucleata showing the spherical central capsule covered by a dark opaque layer and surrounded by an alveolated extracapsular material. Bar = 0.4 mm. B. A drawing of T. maculata by Ernst Haeckel (from Haeckel 1887, Pl. 1 Fig.4). After Haeckel: c, The central capsule; v, vacuoles filling this capsule; n, the central nucleus; l, the concentric nucleolus; g, the voluminous calymma, a small radial piece of which is only presented; a, the large alveoles; b, peculiar exoplasmatic bodies; p, black pigment in the inner zone; f, the retracted pseudopodia in the outer zone of the calymma. C. A drawing of T. melacapsa also by Ernst Haeckel (from Haeckel 1887, Pl. 1 Fig. 5). After Haeckel: n, The large nucleus; l, numerous small nucleoli inside the nucleus; v, the vacuoles filling up the central capsule and separated by black pigment; a, large alveoles of the calymma; k, oil globules; b, exoplasmatic bodies; f, the retracted pseudopodia in the outer zone of the calymma.







swarmer release, therefore greatly enhances amplification of host DNA for further molecular analyses.

## MATERIAL AND METHODS

Thalassicolla nucleata cells were collected in glass jars by divers. Cells were maintained in 0.22  $\mu$ m Millipore-filtered sea water in glass culture tubes and fed brine shrimp (Artemia salina) as food. T. nucleata samples were collected in the Sargasso Sea approximately 4 miles off the southeast coast of Bermuda and in the North Pacific Central Gyre along a transect from Portsmouth, Oregon to Honolulu, Hawaii.

Central capsules of the polycystine radiolarian cells which contain the nucleus, as well as other cellular machinery, were physically separated from extracapsular material which contained endosymbiotic algae at a time in their life cycle immediately before swarmer release. The T. nucleata sequence derived from the Sargasso Sea sample designated TnucBBS 3 was obtained from central capsules of two individuals. The Thalassicolla sequences obtained from the Pacific, designated TnW10.79, TnW10.74, TnW10.72, and TnW10.10, were four different clones derived from a single sample which contained 17 pooled central capsules. Total DNA from Sargasso Sea-collected specimens was extracted, rDNA was amplified, cloned and sequenced as described in Chapter 1. Pacific collected T. nucleata were extracted using the same protocols as the Sargasso Sea-collected specimens. However, amplified rDNA (after Saiki et al. 1988) from Pacific samples was cloned into a double-stranded TA plasmid vector pCRII (Invitrogen) and purified plasmid template DNA for sequencing was obtained using the Magic MiniPrep system (Promega). To minimize sequencing error, double stranded sequence of both the entire forward and reverse strands of the rDNA coding regions was obtained using the Sequenase version 2.0 kit and methods or Sequitherm (Epicentre) kit and Li-Cor automated sequencing methods (Li-Cor).

The 16S-like rRNA sequences of Thalassicolla were aligned against a larger eukaryotic data set by eye with regard to primary and secondary structural conservation using the

Olsen Multiple Sequence Alignment Editing program (Olsen et al. 1992). Absolute percent differences were calculated as a percentage of dissimilarity between pairs of the five T. nucleata sequences. Percent dissimilarity values were obtained by dividing the absolute number of base pair differences between pairs of taxa by the length of the longer sequence of the pair, counting gaps and ambiguities as a single difference, and representing the resulting value as a percentage of 100.

## RESULTS

The SSU rRNA sequences for five representatives of T. nucleata are listed in Fig. 2. Based on this alignment, T. nucleata sequences showed variability at 66 positions scattered over the entire length of the gene. The percent dissimilarity values of these sequences are listed in Table 1. The amount of genetic variation found among samples of T. nucleata small-subunit rRNA gene ranged from 0.45% to 2.54%. The largest dissimilarity values of 2.54% were seen between the sequence from the Sargasso and two sequences from the Pacific sample. The sequences derived from the Pacific sample were more similar to each other than any of the four were to the sequence derived from Sargasso. The gene lengths in base pairs (bp) for the sequences presented in this paper are as follows: TnucBBS3, 1770 bp; TnW10.79, 1771 bp; TnW10.74 , 1765 bp; TnW10.72, 1771 bp.; TnW10.10, 1771 bp.

## DISCUSSION

The degree of variability seen in the T. nucleata sequence data exceeds that expected within a given species and is comparable to that seen between different genera or within genera of other protistan taxa in the literature (Sogin et al. 1986; Manhart et al. 1995). While all the specimens used in this study fit the morphological description of T. nucleata, it is possible that different strains of T. nucleata exist which cannot be distinguished based on morphological criteria. Alternatively, the individuals collected as T. nucleata may have included other species indistinguishable from T. nucleata at the light microscope level. Yet

Table 1. Percent dissimilarity for rDNA sequences derived from different samples of Thalassicolla nucleata.

| Sample #'s | BBS3 | W10.79 | W10.74 | W10.72 | W10.10 |
|------------|------|--------|--------|--------|--------|
| BBS3       | 0    | 2.37   | 2.32   | 2.54   | 2.54   |
| W10.79     |      | 0      | 1.98   | 0.45   | 0.62   |
| W10.74     |      |        | 0      | 1.92   | 2.03   |
| W10.72     |      |        |        | 0      | 0.62   |
| W10.10     |      |        |        |        | 0      |

Fig. 2. The alignment of 16S-like rRNA sequences of Sargasso Sea-collected T. nucleata (TnucBBS3) and four sequences derived from a pooled sample of T. nucleata from the Pacific (TnW10.79, 74, 72 and 10). Sequence identity is represented by dots and nucleotide abbreviations follow the IUB code.

|          |     |  |
|----------|-----|--|
| TnucBBS3 | 1   | AACCUGGUUGAUCCUGCCAGUAGUCAUACGCUAACAUAUAAAGAUUAAGC |
| TnW10.79 | 1   | .....  |
| TnW10.74 | 1   | .....  |
| TnW10.72 | 1   | .....  |
| TnW10.10 | 1   | .....  |
| TnucBBS3 | 50  | CAUGCAUGUACGAGUAUACAAUUAACCAUUUAAACUGCGUAAAGCUCAU  |
| TnW10.79 | 50  | .....A.A....U.....                                 |
| TnW10.74 | 50  | .....A.--.....                                     |
| TnW10.72 | 50  | .....A.A....U.....                                 |
| TnW10.10 | 50  | .....A.A....U.....                                 |
| TnucBBS3 | 99  | UAUAUCAGUUCUAAACAUUUAGGAAUACAAAAAAGAUGGAUAUUAGUGC  |
| TnW10.79 | 99  | .....G-....A.C.....                                |
| TnW10.74 | 97  | .....U-....A.C.....                                |
| TnW10.72 | 99  | .....G-....A.C.....                                |
| TnW10.10 | 99  | .....G-....A.C.....                                |
| TnucBBS3 | 148 | UAAUUCUACAAUCAAUACAUUUUAACGUCUAAUUUU-UUAGACUAAAU   |
| TnW10.79 | 147 | .....U....AA....U.....                             |
| TnW10.74 | 145 | .....A.-....U.....                                 |
| TnW10.72 | 147 | .....U....AA....U.....                             |
| TnW10.10 | 147 | .....U....AA....U.....                             |
| TnucBBS3 | 196 | UACUGAGUAUCAAAAAGUACGACUAUCUGAAUUCUAAUAUUUACUGGUU  |
| TnW10.79 | 196 | .....G.....  |
| TnW10.74 | 193 | .....  |
| TnW10.72 | 196 | .....G.....  |
| TnW10.10 | 196 | .....G.....  |
| TnucBBS3 | 245 | ACACUACAGAGUGAUAGUUCUAUUUAGUGACUGACCCAUCAGUUGUUCU  |
| TnW10.79 | 245 | .....  |
| TnW10.74 | 242 | .....  |
| TnW10.72 | 245 | .....  |
| TnW10.10 | 245 | .....  |
| TnucBBS3 | 294 | AUUAUGUAGUGAAUUAUUGAGGCUGAAACGGGUAGCGGAGAAUAGGGU   |
| TnW10.79 | 294 | .....  |
| TnW10.74 | 291 | .....  |
| TnW10.72 | 294 | .....  |
| TnW10.10 | 294 | .....  |
| TnucBBS3 | 343 | UCCGUUCCGGAGAAAAGAGCCUGCGAAACGGCUACUACAUCUAAGGAAGG |
| TnW10.79 | 343 | .....  |
| TnW10.74 | 340 | .....  |
| TnW10.72 | 343 | .....  |
| TnW10.10 | 343 | .....  |
| TnucBBS3 | 392 | CAGCAGGCGNGUAAAUAUUCAAUUCUAAAUCAGAGAGUUAGUAACAAU   |
| TnW10.79 | 392 | .....C.....  |
| TnW10.74 | 389 | .....C.....  |
| TnW10.72 | 392 | .....C.....C.....                                  |
| TnW10.10 | 392 | .....C.....  |

|          |     |  |
|----------|-----|--|
| TnucBBS3 | 441 | AUAUUACGAUGUUAAACCUUAGGGUUAUUUAUUUAUUGAGGAUAGAU    |
| TnW10.79 | 441 | .....  |
| TnW10.74 | 438 | .....  |
| TnW10.72 | 441 | .....  |
| TnW10.10 | 441 | .....  |
| <br>     |     |  |
| TnucBBS3 | 490 | UAUUUAUUUAUACGAUUGACUAUAGGAGGGCAAGUCUGGUGCCAGCAGC  |
| TnW10.79 | 490 | .....  |
| TnW10.74 | 487 | .....A.....  |
| TnW10.72 | 490 | .....  |
| TnW10.10 | 490 | .....  |
| <br>     |     |  |
| TnucBBS3 | 539 | CGCGGUAAUACCAGCUCCAAUAGUGUAUGCUAACGUUGUUGCAGUUACA  |
| TnW10.79 | 539 | .....  |
| TnW10.74 | 536 | .....  |
| TnW10.72 | 539 | .....  |
| TnW10.10 | 539 | .....  |
| <br>     |     |  |
| TnucBBS3 | 588 | AAGCUCGUAGUUGGUCUAUUAUGARUUUUUUUAUUAUAGUAUAAUUG    |
| TnW10.79 | 588 | .....A.....  |
| TnW10.74 | 585 | .....A.....G.....                                  |
| TnW10.72 | 588 | .....A.....  |
| TnW10.10 | 588 | .....A.....  |
| <br>     |     |  |
| TnucBBS3 | 637 | UACUAUUUAUUGACAAUGCCUAAAUCUUACUUAGAACAUGUACUAUGUUG |
| TnW10.79 | 637 | .....CU...A...                                     |
| TnW10.74 | 634 | C.....C.....U...A...                               |
| TnW10.72 | 637 | .....CU...A...                                     |
| TnW10.10 | 637 | .....CU...A...                                     |
| <br>     |     |  |
| TnucBBS3 | 686 | AAAYUUACUUUUCGACAUUCCUCAUGUUUGUUAUUACUUUGAAAAAAU   |
| TnW10.79 | 686 | ...U.....U.....U.....                              |
| TnW10.74 | 683 | ...U...U.....U.....                                |
| TnW10.72 | 686 | ...U.....U.....U.....                              |
| TnW10.10 | 686 | ...U.....U.....U.....                              |
| <br>     |     |  |
| TnucBBS3 | 735 | UAUGUUGAUUUUAAGAGAAGAAAUGAUUUGUACUAUAGUACAGAAUAAU  |
| TnW10.79 | 735 | .....  |
| TnW10.74 | 732 | .....C...  |
| TnW10.72 | 735 | .....  |
| TnW10.10 | 735 | .....C.....  |
| <br>     |     |  |
| TnucBBS3 | 784 | ACUUGAAGAUCUCAGUAAAACUAAAACUUUUGGAUACUGGUGUARUGCU  |
| TnW10.79 | 784 | .....G.....A.....                                  |
| TnW10.74 | 781 | .....A.....  |
| TnW10.72 | 784 | .....G.....A.....                                  |
| TnW10.10 | 784 | .....G.....A.....                                  |
| <br>     |     |  |
| TnucBBS3 | 833 | CUUUAGAGUUAGCUGAAGAUAAUAAUAAUUUAGCGWUAGAGGUGAAAAUU |
| TnW10.79 | 833 | .....U.....  |
| TnW10.74 | 830 | .....U.....  |
| TnW10.72 | 833 | .....U.....  |
| TnW10.10 | 833 | .....U.....  |

|          |      |   |
|----------|------|---|
| TnucBBS3 | 882  | CAAGAAUCGUUAUAAGAUUAACAAGUGCCAAAGCAAUUAUCUAAGAUUA |
| TnW10.79 | 882  | U.....  |
| TnW10.74 | 879  | .....U.....                                       |
| TnW10.72 | 882  | .....   |
| TnW10.10 | 882  | .....A.....                                       |
| TnucBBS3 | 931  | AUUCAUUGAUCAAGAACGUAAGUUGAAGGAUUGAAGACGAUCAGAUACC |
| TnW10.79 | 931  | .....   |
| TnW10.74 | 928  | .....   |
| TnW10.72 | 931  | .....   |
| TnW10.10 | 931  | .....   |
| TnucBBS3 | 980  | GUCGUAAUCUCAAUUGUAAACUAUAUCAACUAGGGAUUAAACACUGUUU |
| TnW10.79 | 980  | .....   |
| TnW10.74 | 977  | .....   |
| TnW10.72 | 980  | .....   |
| TnW10.10 | 980  | .....   |
| TnucBBS3 | 1029 | UUUAUGACAUGUUGGCACCUUGUGAGAAAUUAGAGUUCUCAGAUUCCG  |
| TnW10.79 | 1029 | .....   |
| TnW10.74 | 1026 | C.....  |
| TnW10.72 | 1029 | .....   |
| TnW10.10 | 1029 | .....   |
| TnucBBS3 | 1078 | GGGGGAGUAUGGUUGCAAGUCUGAAACUUAAGGAAUUGACGGAAGGGC  |
| TnW10.79 | 1078 | .....   |
| TnW10.74 | 1075 | .....   |
| TnW10.72 | 1078 | .....   |
| TnW10.10 | 1078 | .....   |
| TnucBBS3 | 1127 | ACCACAAGUUGUGGAUACUGUGGCUUAAUUUGACUCAACACUGGAAAAC |
| TnW10.79 | 1127 | .....   |
| TnW10.74 | 1124 | .....   |
| TnW10.72 | 1127 | .....   |
| TnW10.10 | 1127 | .....   |
| TnucBBS3 | 1176 | UUACCAGGUCCAGACAUAUUUAGGAUUGACAGAUUAAUAGCCCUGUCCU |
| TnW10.79 | 1176 | .....GC.....                                      |
| TnW10.74 | 1173 | .....   |
| TnW10.72 | 1176 | .....GC.....                                      |
| TnW10.10 | 1176 | .....GC.....                                      |
| TnucBBS3 | 1225 | GAUUUUGUGGCUGGUGGUGCAUGGCCGUUCUAGUUGGUGAAGUGAUUU  |
| TnW10.79 | 1225 | .....   |
| TnW10.74 | 1222 | .....   |
| TnW10.72 | 1225 | .....G.....                                       |
| TnW10.10 | 1225 | .....   |
| TnucBBS3 | 1274 | GUCUGGUUUAUCCGUUAACGAACGAGACUAUUACCAAUAAUAGUAAG   |
| TnW10.79 | 1274 | .....U.....                                       |
| TnW10.74 | 1271 | .....   |
| TnW10.72 | 1274 | .....   |
| TnW10.10 | 1274 | .....   |

|          |      |  |
|----------|------|--|
| TnucBBS3 | 1323 | YACUGCA--UUAGCAGUGUGAUUACUUCUUAGAGGGACUGGUGAUACAU  |
| TnW10.79 | 1323 | CG...U.UU..C.....                                  |
| TnW10.74 | 1320 | U....U-.A...A.....                                 |
| TnW10.72 | 1323 | CG..AUGUU..C.....                                  |
| TnW10.10 | 1323 | CG...UGUU..C...C.....                              |
|          |      |  |
| TnucBBS3 | 1370 | AAGUUACUGGAGGCAAGUUGCAAUGACAGGUCUGUGAUGCCCUUAGAUG  |
| TnW10.79 | 1372 | .....  |
| TnW10.74 | 1368 | .....  |
| TnW10.72 | 1372 | .....  |
| TnW10.10 | 1372 | .....  |
|          |      |  |
| TnucBBS3 | 1419 | UACUGGGCCGCGCACGGGAUACAACAGGGGAGAUAAUAUGUACAUUUAA  |
| TnW10.79 | 1421 | .....U.....A.....-..                               |
| TnW10.74 | 1417 | .....U.....A.....-..                               |
| TnW10.72 | 1421 | .....U.....A.....-..                               |
| TnW10.10 | 1421 | .....U.....A.....-..                               |
|          |      |  |
| TnucBBS3 | 1468 | ACAUAUUUUGACAAUAAUAUUGUAACCGYGAAUCUGUCUUUAAUAUGGA  |
| TnW10.79 | 1469 | .....U.....C.....                                  |
| TnW10.74 | 1465 | .....U.....C.....                                  |
| TnW10.72 | 1469 | .....U.....C.....                                  |
| TnW10.10 | 1469 | .U.....U.....C.....                                |
|          |      |  |
| TnucBBS3 | 1517 | AUUGCACUAUGCAAUUUUACACAUAACUAGGAAUAUCUUGUAAGUACA   |
| TnW10.79 | 1518 | .....G.....  |
| TnW10.74 | 1514 | .....  |
| TnW10.72 | 1518 | .....G.....  |
| TnW10.10 | 1518 | .....G.....  |
|          |      |  |
| TnucBBS3 | 1566 | UGUCAUAAUCGUGUUCUGAAUGCGUCCUGUCCUUUGUACACACCGCCCC  |
| TnW10.79 | 1567 | .....  |
| TnW10.74 | 1563 | .....C.....  |
| TnW10.72 | 1567 | .....  |
| TnW10.10 | 1567 | .....  |
|          |      |  |
| TnucBBS3 | 1615 | GUCGCUCCUACCGAUUGGAUGAGAUGGUGAGUAAAUCUUAUAUGAUUGAA |
| TnW10.79 | 1616 | .....  |
| TnW10.74 | 1612 | .....  |
| TnW10.72 | 1616 | .....  |
| TnW10.10 | 1616 | .....C.....  |
|          |      |  |
| TnucBBS3 | 1664 | GUUAUACUGUAAAAGUUGAAKGUCAGUUAUAUAUAUUUGCAAACUAAACU |
| TnW10.79 | 1665 | ....A.....U....A..G.....                           |
| TnW10.74 | 1661 | .....U.....G.....                                  |
| TnW10.72 | 1665 | ....A.....U.....G.....                             |
| TnW10.10 | 1665 | .....U....A..G.....                                |
|          |      |  |
| TnucBBS3 | 1713 | AUUUAGAGGAAGGAGAAGUCGUAACAAGGUUCCGUAGGUGAACCUGCA   |
| TnW10.79 | 1714 | .....  |
| TnW10.74 | 1710 | .....U.....  |
| TnW10.72 | 1714 | .....U.....  |
| TnW10.10 | 1714 | .....  |



|          |      |           |
|----------|------|-----------|
| TnucBBS3 | 1762 | GAAGGAUCA |
| TnW10.79 | 1763 | .....     |
| TnW10.74 | 1759 | .....     |
| TnW10.72 | 1763 | .....     |
| TnW10.10 | 1763 | .....     |

a third possibility is that T. nucleata possesses multiple copies of its SSU rRNA genes which differ in both length and base pair composition. The last of these three possibilities is difficult to address because individuals were pooled when samples were collected. This would make it impossible to determine the source of heterogeneity (e.g. interspecific vs. intraspecific variability). The first two possibilities require a better understanding of the criteria used in defining species of Thalassicolla and are addressed below.

In reviewing the original species descriptions made by Haeckel, it seems likely that at least some of Haeckel's species were probably descriptions of different physiological states of a given species or descriptions of individuals infected by parasitic dinoflagellates. For example, dinoflagellate infections are known to occur in T. nucleata (Chatton 1920; Hollande 1974) and were observed during this study in a number of T. nucleata specimens that were not observed to undergo swarmer formation but instead erupted with dinoflagellate parasites. In all cases, such individuals of T. nucleata lacked symbionts and possessed a yellowish-orange color to the central capsule which was visible beneath the dark covering of the central capsule. While all of these infected T. nucleata specimens (possessing yellowish-orange central capsules) were observed in the Sargasso, Haeckel makes similar references to cell-types with such yellowish-colored central capsules in T. nucleata (which is a cosmopolitan species) and also in another species in the Pacific (namely, T. maculata Fig. 1B). These details are noteworthy because of the prevalence with which we encountered specimens of this description during various trips to the Sargasso Sea. In addition, Haeckel described T. maculata as possessing no zooxanthellae. It seems possible that parasitism may also occur in the Pacific and that T. maculata is just a description of a stage in the parasitism of T. nucleata. Although parasitism appears to occur in Thalassicolla collected from other geographic locations, information for its frequency in the Pacific is poorly documented.

The presence or absence of an opaque layer surrounding the central capsule rendering it colorless was another criterion Haeckel used to define species of Thalassicolla. It is noteworthy that in two of the species descriptions of Thalassicolla in which Haeckel described members with colorless central capsules (T. pellucida and T. australis), he also reported an absence of zooxanthellae. This is interesting because complete loss of the extracapsular material can happen when an individual is sufficiently agitated, as might occur during ingestion, excessive wave action or excessive agitation in net tows (Verworm 1891; Gamble 1909; O. R. Anderson, personal communication). When individuals shed their dark extracapsular material, they also shed their symbionts and may require some time before regenerating the opaque layer and acquiring a new population of symbionts.

The size of the central capsule also has been used by Haeckel to delineate species of Thalassicolla, as in his description of T. melacapsa (Fig. 1C). Likewise, this feature is a questionable taxonomic criterion because of possible variability originating from non-genetic origins. For example, the diameter of the central capsule in Thalassicolla has been observed to change within an individual, possibly in response to physiological condition (O. R. Anderson, Amaral Zettler, personal observation). Furthermore, many of Haeckel's descriptions make reference to the "patchy" appearance of the opaque-layer surrounding the central capsule, however, this characteristic may also be attributed to nutritional status and variation in light intensity (O. R. Anderson, personal observation).

Since we lack type specimens and even drawings of all of the original species of Thalassicolla described by Haeckel, it is impossible to determine whether or not the above observations are important in determining the actual number of species for the genus. We do not consider this to be an exhaustive study of the species diversity of the genus Thalassicolla. However, we obtained notable differences at the level of the SSU rRNA gene which raises the question of what defines the species T. nucleata. Due to the manner in which these samples were collected it is impossible to know if these differences

represent intraspecific (multiple alleles of the rDNA gene within one species) or interspecific variability (different genes of different species). This question could be addressed by examining a single individual or preferably several individuals separately. With the current sequence information in hand, genus specific-primers could be designed to further explore the extent to which the morphological criteria used in Haeckel's species designations reflect reliable phenotypic markers for distinguishing between different species of Thalassicolla.

#### ACKNOWLEDGMENTS

This material is based upon work supported under a National Science Foundation Minority Graduate Fellowship. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation. Further financial support is acknowledged in part from NSF (OCE-9314533) (to D. A. Caron) and from the Scurlock Fund for research at the Bermuda Biological Station for Research, Inc. (BBSR), the Grant-In-Aid Fund of BBSR, funds from the Woods Hole Coastal Research Center, the Education Office of the Woods Hole Oceanographic Institution and the Lerner Gray Fund for Marine Research of the American Museum of Natural History (to L. A. Amaral Zettler). We wish to thank the Dr. Gregory Hinkle for expert training in molecular techniques during the early stages of this work. The authors also wish to acknowledge the assistance of Dr. Anthony Michaels of the Bermuda Biological Station for opening up his lab and allowing lab members especially Frances Howes and numerous other volunteers to assist with blue water diving collection of specimens on a regular basis.

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## **Chapter 3**

### **Towards a Molecular Phylogeny of Colonial Spumellarian Radiolaria<sup>1</sup>**

<sup>1</sup> The classification scheme of Strelkov and Reshetnyak (1971) was used for classifications at the family-level and below and that of Levine et al. (1980) for higher-level classifications.





ABSTRACT. Throughout their history of classification, the colonial spumellarian radiolaria have been grouped together taxonomically on the basis of their ability to form colonies. A molecular phylogenetic basis for this grouping, however, has never been explored. We used small-subunit ribosomal RNA gene sequence data to examine whether the colonial spumellarian radiolaria (Polycystinea) constitute a monophyletic evolutionary assemblage. Representatives from two spumellarian families known to form colonies, the Sphaerozoidae and the Collosphaeridae were considered in this study and included the following taxa: Sphaerozoidae: Collozoum pelagicum; Collozoum serpentinum; Rhaphidozoum acuferum; Sphaerouzoum punctatum; and Collosphaeridae: Collosphaera globularis-huxleyi; Acrosphaera (circumtexta?); and Siphonosphaera cyathina. The results from our molecular phylogenetic analyses do not strongly support the monophyly of the colonial spumellarian radiolaria yet do not completely eliminate this possibility either. Coloniality may have arisen more than once among the Spumellarida or existing solitary Spumellarida may have once possessed colonial forms. All molecular analyses supported the monophyly of the Collosphaeridae but only distance analyses supported the monophyly of the Sphaerozoidae. The idea that coloniality appeared more than once in spumellarian evolution is contrary to current opinion based on skeletal morphogenesis studies but has been suggested from studies of the fossil record.

Supplementary key words. Acrosphaera, Collosphaeridae, Collozoum, colonial radiolaria, planktonic sarcodine, Siphonosphaera, Sphaerozoidae

Colonial spumellarian radiolaria are holoplanktonic sarcodines (Subphylum Sarcodina, Class Polycystinea) which occur exclusively in open ocean oligotrophic environments. As in all polycystines, each cell is physically separated into the endocyttoplasm and the ectocyttoplasm by a porous proteinaceous capsular wall. The capsular wall, together with the major cellular machinery it encloses, (the nucleus, mitochondria, golgi, endoplasmic reticulum, vacuoles, and oil droplets), is referred to as the central capsule. In colonial spumellarian radiolaria, thousands of individual central capsules extend their pseudopodia into a shared gelatinous extracapsular matrix which connects the cells and also typically houses numerous symbiotic algae.

As "multicellular" entities, the colonial spumellaria are macroscopic and have been reported to reach lengths of up to three meters, making them very conspicuous components of tropical and subtropical pelagic marine environments (Anderson and Swanberg 1981). Despite a visible presence in the plankton, their fragile nature and resistance to laboratory culture has left many unanswered questions regarding colonial spumellarian biology, including the reasons for colony formation. Apart from isolated reports of colony formation by phaeodaria of the family Tuscaroridae (Haecker 1908; Swanberg 1979), the spumellaria are the only other "radiolaria" sensu stricto (Polycystinea and Phaeodarea) which form colonies.

The "colonial radiolaria" are restricted to two families within the order Spumellarida; the Sphaerzoidae and the Collosphaeridae. In the Sphaerzoidae, skeletal material is either lacking or else composed of several silicate spicules of varying degrees of complexity. The most recent systematic treatment of the colonial spumellaria (Strelkov and Reshetnyak 1971) divides the Sphaerzoidae into three genera, Collozoum, Sphaerzoum, and Rhaphidozoum. The genus Collozoum possesses either simple spines (Strelkov and Reshetnyak 1971) or no skeleton. Members of the genus Sphaerzoum contain characteristic paired-triradiate spines, while Rhaphidozoum representatives have both

simple and radiate spines. Species designations are typically based on the structure of these spines, when present, or the morphology of the central capsular wall, as in the case of species within the genus Collozoum.

All members of the family Collosphaeridae are characterized by siliceous, spherical latticed shells having varying degrees of ornamentation. Strelkov and Reshetnyak (1971) divided the Collosphaeridae into three tribes, the Collosphaerini, the Acrosphaerini and the Siphonosphaerini, in order to maintain a more "natural" system of classification. In brief, Collosphaerini possess smooth surfaces on both the inner and outer portions of the shell, Acrosphaerini have a spine-covered outer surface of the latticed shell, and the Siphonosphaerini have latticed shells whose pores are either partially or completely elongated into tube-like projections.

It is generally assumed that the members of the colonial spumellaria were derived from a single common ancestor and that the ability to form colonies has arisen only once in their evolution (Strelkov and Reshetnyak 1971; Anderson and Swanberg 1981). Some authors have suggested that colonial spumellaria are part of a life cycle stage of solitary forms which undergo multiple binary fission of their central capsule to form colonies or perhaps that they are different stages of the same species (Brandt 1902; Hollande and Enjumeat 1953; Swanberg 1979). Solitary forms are, in fact, known for some members of the Sphaerozoidae. The genus name Thalassophysa, for example, is used when referring to the solitary stage of the various members of Collozoum. In the taxa examined in this study, Thalassophysa sanguinolenta is the name given to the solitary stage of the colonial Collozoum pelagicum (Brandt, 1902). C. serpentinum is also recognized as having a solitary stage (Swanberg, 1979). Solitary forms have, however, never been observed for members of the Collosphaeridae. While members of the Collosphaeridae have left behind a fossil record, only individual shells are found in the marine sediments. Therefore, it is unknown whether or not fossil collosphaerids also produced colonies but it is assumed that

they did. Likewise, we are working under the assumption that Thalassicolla has evolved from a solitary ancestor and that the genus is not capable of forming colonies. All available information in the literature and personal observation indicates that the genus is strictly solitary, however, the possibility that Thalassicolla evolved from a colonial ancestor and has now lost the character of coloniality, cannot be excluded.

Due to the application of molecular biological techniques, scientists now have a novel means of exploring the question of coloniality in spumellarian evolution. We sequenced the small-subunit ribosomal RNA (SSU rRNA) genes of representatives from both families of Spumellarida known to form colonies in order to examine the origins of coloniality and investigate the evolutionary relationships among the colonial spumellaria.

#### MATERIAL AND METHODS

Colonial spumellarians were collected in glass jars by divers. Colonies were maintained in 0.22  $\mu\text{m}$  Millipore-filtered seawater in glass culture tubes with brine shrimp (Artemia salina) as food. All samples were collected approximately 4 miles off the southeast coast of Bermuda on the dates listed below. Samples were typically given individual sample designations prior to identification. The following samples were included in this study, with sample designation and collection date following the species identification: Collozoum pelagicum (BBSR 2, November, 1993); Rhaphidozoum acuferum (BBSR 7, November, 1993); Collosphaera globularis-huxleyi (BBSR 173, May, 1994); Sphaerozoum punctatum (CR 4, May, 1995); Acrosphaera (circumtexta?) (CR 6, May, 1995); Collozoum serpentinum (CR 16, May, 1995); Siphonosphaera cyathina (October, 1995). C. pelagicum consisted of a section of a vegetative (non-reproductive) colony. All other samples consisted of pooled or single central capsules from a single reproductive colony.

In all but the C. pelagicum sample, colonies were held until the early stages of onset of swarmer production. At that time, central capsules were physically separated from extracapsular material which contained endosymbiotic algae by repeated micropipeting.

The rationale behind sacrificing individuals at that point in their life cycle was twofold: first, a natural amplification of DNA occurs within the organism at that time as multiple copies of the genome are made in preparation for swarmer formation. Second, many species either consume or expel endocyttoplasmic symbiotic algae immediately prior to swarmer formation thereby reducing the potential of amplifying non-target DNA. Individual central capsules were pipetted through several 0.22  $\mu$ m-Millipore filtered seawater rinses followed by a final MilliQ-water rinse prior to placement in a modified 1X PCR buffer solution which consisted of 50 mM KCl, 10 mM Tris, pH 8.3, 2 mM MgCl<sub>2</sub>, 0.001% Gelatin, and 1.0% NP40 (Sigma; St. Louis, MO). Samples were then stored frozen at -70°C. Samples used for molecular analyses were heated at 95°C for 10 minutes to lyse cells and liberate DNA. An aliquot of the lysed sample was used directly in PCR amplification reactions (Saiki et al. 1988). Sequences from R. aciferum and C. globularis-huxleyi samples were obtained from cloned products (Chapter 1). Sequence information obtained from these two samples was then used to design "colonial spumellarian"-specific primers which were effective in amplifying SSU rRNA genes of different genera.

Sequence data from the remaining samples were obtained from directly sequencing PCR products amplified using a combination of colonial spumellarian specific primers and Medlin primers (Medlin et al. 1988). These colonial spumellarian-specific primers were synthesized as described in Chapter 1. The nucleotide sequences are: forward primer R906, 5'-TATTAGTATTTTTRTCGTT-3'; reverse primer R1451bio, 5'-TATTGTAG-CCCGTGCGCT-3' (previously used as a probe in in situ verification experiments in Chapter 1). PCR reactions consisted of 3 minutes of denaturation at 95°C followed by 30 amplification cycles each consisting of 94°C for 1 minute, 42°C for 1 minute and 72°C for 2 minutes. Two separate 100  $\mu$ l PCR reactions typically provided enough template for sequencing reactions. PCR reactions were then pooled prior to purification using the Wizard PCR Kit (Promega; Madison, WI) to obtain purified DNA for direct sequencing.

Direct sequencing of PCR products was accomplished using IR-labeled primers and reagents from the Sequitherm Long-Read Sequencing Kit (Sequitherm; Madison, WI), along with the Sequitherm Cycle sequencing protocol developed by Li-Cor (Lincoln, NE) which consisted of 5 minutes of denaturation at 95°C prior to 30 cycles of 20 sec at 95°C (30 sec for plasmid DNA), 30 sec at 60°C, and 1 minute at 70°C using a Perkin Elmer 2400 thermo-cycler. Double stranded sequencing of the entire forward and reverse strands of the rDNA coding regions was conducted for cloned products. For directly-sequenced PCR products, double-stranded read for the all but the primer-specified ends were obtained.

The 16S-like rRNA sequences of colonial spumellaria were aligned against a subset of the total eukaryotic alignment data base (Olsen et al. 1992). Sequences were aligned by eye using the Olsen Multiple Sequence Alignment Editing program with regard to primary and secondary structural conservation. The same positions were used in this analysis as were used in the data set analyzed in Chapter 1 (1,368 total sites minus one site which became a gap when certain taxa were removed). In addition to colonial spumellaria, the alignment also included the solitary spumellarian Thalassicolla nucleata (Chapter 1) and acantharian outgroups Haliommatidium sp. and Chaunacanthid 218 (Chapter 1). In reality, however, no clearly appropriate outgroups exist for the spumellaria at the time of the writing of this manuscript since the spumellarian sequences are extremely divergent and are unrelated to any other taxa for which SSU rRNA sequence data is available.

The colonial spumellarian sequences were also analyzed independently of an outgroup (in "unrooted" networks) in order to include more sites in the analysis (an expanded number of homologous sites which included 1,635 positions). Molecular phylogenetic relationships were inferred for both data sets using distance (Olsen 1988), maximum parsimony (Swofford 1991) and maximum likelihood (Olsen et al. 1994) methods. The robustness of the tree topologies obtained were examined using 100 bootstrapping resamplings for all

three methods and additionally for the maximum parsimony method using decay analyses (Bremer 1988).

The decay analyses were accomplished by first doing an exhaustive search using PAUP 3.1.1 (Swofford, 1991) to obtain the length of the most parsimonious tree, and then sequentially adding steps to the value of the shortest tree found using the initial upper bound setting of the branch and bound search option. Resulting trees constructed at each additional step-allowance were then consensed in a strict consensus tree, and the order in which various clades "decayed" was compared.

## RESULTS

Photomicrographs of the skeletal structures of spicule-bearing and skeleton-bearing colonial spumellaria used in this study are shown in Fig. 1. Species identifications were straightforward with the following two exceptions. Sample number BBSR 173 was best described as Collosphaera globularis-huxleyi, owing to features of the latticed shell possessed by this specimen (see Fig. 1), which appeared to exhibit qualities shared by both C. globularis and C. huxleyi. Haeckel (1887) asserted that these two species of Collosphaera formed intergrades. Therefore a combined species (globularis-huxleyi) description for this sample seemed most appropriate given the qualities of the shell morphology. Sample number CR 6 is Acrosphaera. The species designation was difficult to ascertain but is probably A. circumtexta. The length in base pairs and % G + C content of the SSU rRNA genes of spumellaria used in this study are listed in Table 1.

The results obtained from the three different phylogenetic methods used in this study did not identify a single common tree (Fig. 2). Distance methods failed to clearly segregate the solitary spumellarian T. nucleata from the colonial spumellaria. Maximum parsimony was the only method which segregated the colonial spumellaria from the solitary spumellarian T. nucleata, with low (61%) but significant bootstrap support. Weak support for the node uniting all the colonial spumellaria was also identified in the parsimony tree by the decay

Table 1. The gene lengths in base pairs (bp) and % G + C content of spumellaria used in this study.

| Species                                | Length (bp) | SSU rDNA |
|--|-------------|----------|
|  |             | % G + C  |
| <u>Thalassicolla nucleata</u>          | 1770        | 36       |
| <u>Rhaphidozoum acuferum</u>           | 1813        | 39       |
| <u>Sphaerozoum punctatum</u>           | 1788        | 37       |
| <u>Collozoum pelagicum</u>             | 1792        | 38       |
| <u>Collosphaera globularis-huxleyi</u> | 1797        | 35       |
| <u>Acrosphaera (circumtexta?)</u>      | 1803        | 35       |
| <u>Siphonosphaera cyathina</u>         | 1791        | 36       |
| <u>Collozoum serpentinum</u>           | 1798        | 38       |





Fig. 1. Photomicrographs of voucher sections of shell-bearing and spicule bearing colonies taken of samples used in this study. A. Acrosphaera (circumtexta?). Note the ridge-like structures often connected with thin bars. The spines, which characterize members of this genus, did not photograph well in this specimen. B. Siphonosphaera cyathina. Note the cylindrical, short tube-like projections which characterize the genus. In S. cyathina the tube-like projections are irregularly dispersed and sometimes terminate with a folded-back distal edge. C. Rhaphidozoum acuferum. This species is characterized by having both simple and radiate spines as the ones shown in this panel. D. Collosphaera globularis-huxleyi. A portion of the latticed-shell of this specimen reveals smooth inner and outer surfaces which characterize members of this genus. This specimen was given a species designation of C. globularis-huxleyi because while most of the pore and bar dimensions matched those reported for C. globularis a small number of specimens possessed shapes more similar to C. huxleyi. E. Sphaerozoum punctatum. This specimen shows the paired triradiate spicules possessed by this genus. The spines of S. punctatum are often barbed as seen in this photograph. Note the numerous crystal inclusions of the swarmers within the central capsule of this reproductive individual. Scale bar = 48  $\mu$ m for all panels.

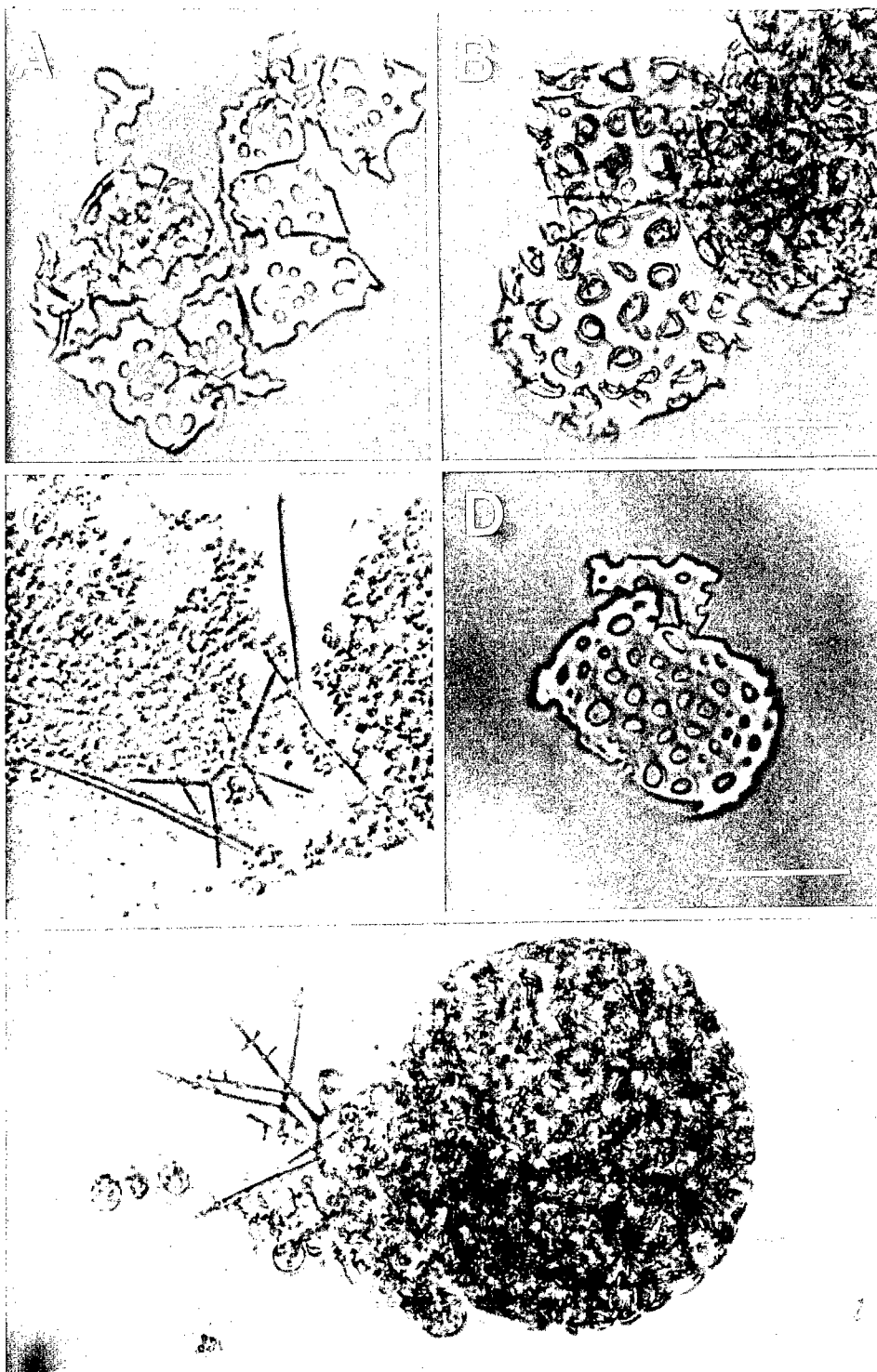
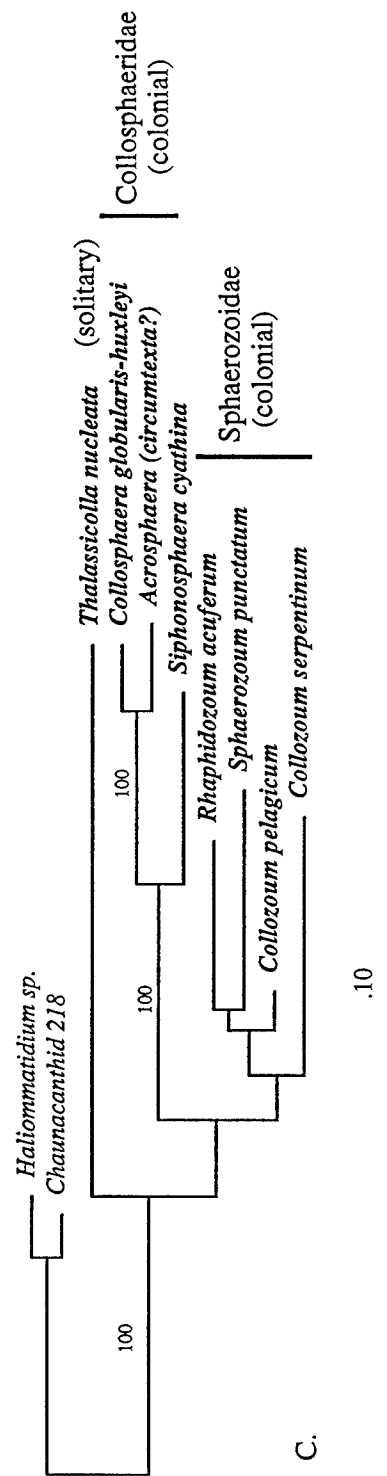
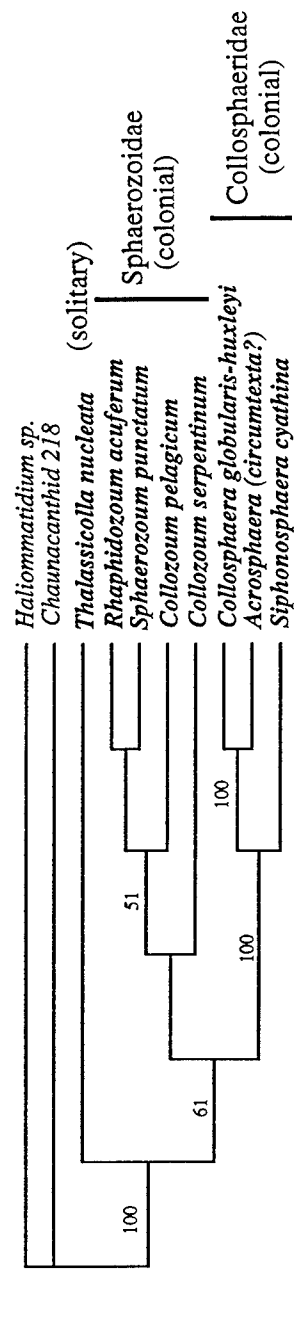
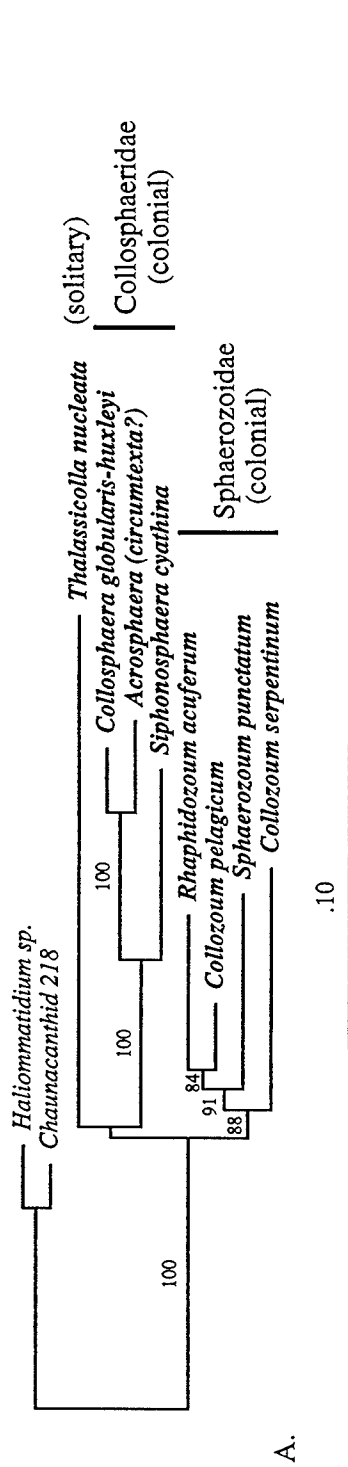






Fig. 2. Phylogenetic reconstructions for solitary and colonial spumellarians using acantharian outgroups Haliommatidium sp. and Chaunacanthid 218 inferred from: **A.** distance, **B.** maximum parsimony and **C.** maximum likelihood methods. There were 1,368 positions used in the phylogenetic analyses. All bootstrap values were computed separately for 100 resamplings of the three respective data sets. Only bootstrap values greater than 50 % are shown. The evolutionary distances are indicated by the bar insert (distance and maximum likelihood) which represents 10 changes per 100 nucleotides.



analysis in which collapse of this node occurred after only 3 steps. Maximum likelihood methods yielded the same topology as maximum parsimony but the branching of T. nucleata separate from the colonial spumellaria was not well-supported by bootstrapping analysis. A likelihood ratio test was conducted (data not shown) but failed to find a significant difference between the distance, maximum parsimony and maximum likelihood tree topologies.

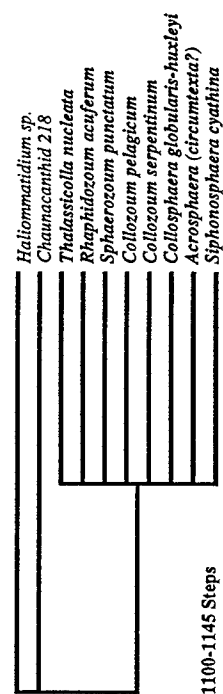
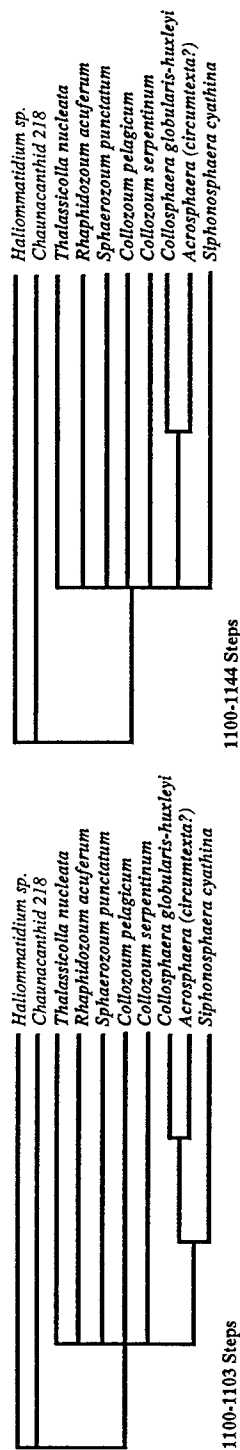
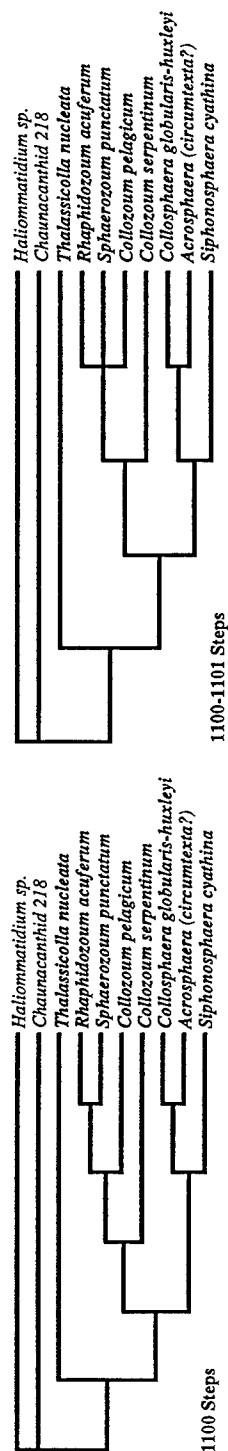
The monophyly of the Collosphaeridae was well supported in all methods for both the analyses with acantharian outgroups (Fig. 2 A - C) and the "unrooted" networks shown in Fig. 4 (A - C) (based on bootstrap values of 100% in all cases). Likewise, in both decay analyses (Figs. 3 & 5), the node leading to the Collosphaeridae was the last to collapse, implying robust support for this clade. The branching pattern within the Collosphaeridae consistently placed S. cyathina branching prior to the divergence of C. globularis-huxleyi and A. (circumtexta?) in all methods used. The strong support for the grouping of C. globularis-huxleyi and A. (circumtexta?) was revealed in the decay analysis of a consensus tree (Fig. 3), in which it required an additional 45 steps before the Collosphaeridae clade completely collapsed.

The separation of the remaining two families (the Sphaerozoidae and the Thalassicollidae) belonging to the suborder Sphaerocollina was not clearly supported in all cases. The bootstrap support values for these latter two families varied dramatically in the distance analysis relative to the maximum parsimony and maximum likelihood analyses (Fig. 2, A, C). The distance analysis clearly isolated the Sphaerozoidae from the Collosphaeridae and T. nucleata (bootstrap value of 88 on the branch leading to the Sphaerozoidae). Although the maximum parsimony and maximum likelihood methods supported a separate ancestry for the Sphaerozoidae distinct from the Collosphaeridae, the low bootstrap support for the parsimony and maximum likelihood tree topologies indicate poor support for the Sphaerozoidae as a distinct clade.





Fig. 3. Results from a decay analysis of the most parsimonious tree obtained from an exhaustive search. The number of additional steps required to produce the consensus trees with progressive degrees of collapse of major nodes is shown to the bottom left of each corresponding tree.



1100-1145 Steps

Fig. 4. Three "unrooted" trees obtained from A. distance, B. maximum parsimony and C. maximum likelihood methods using additional (1,635) positions in analyses. Only bootstrap values greater than 50 % are shown. The evolutionary distances are indicated by the bar insert (distance and maximum likelihood) which represents 10 changes per 100 nucleotides.

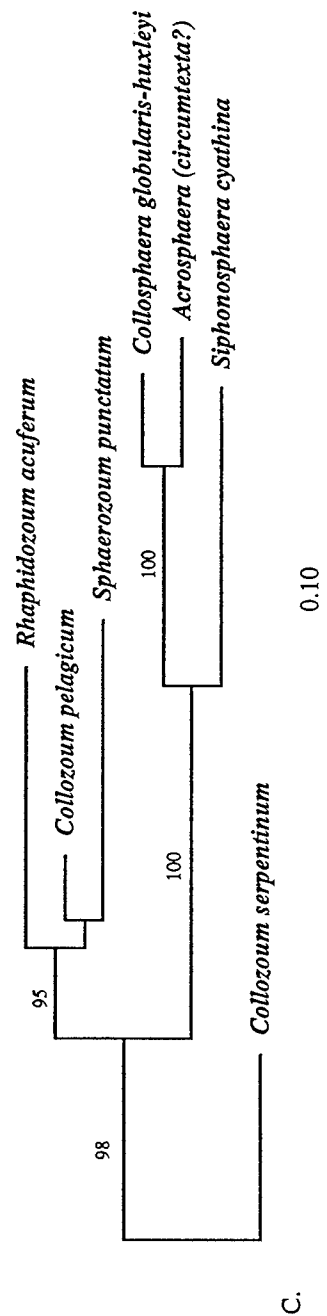
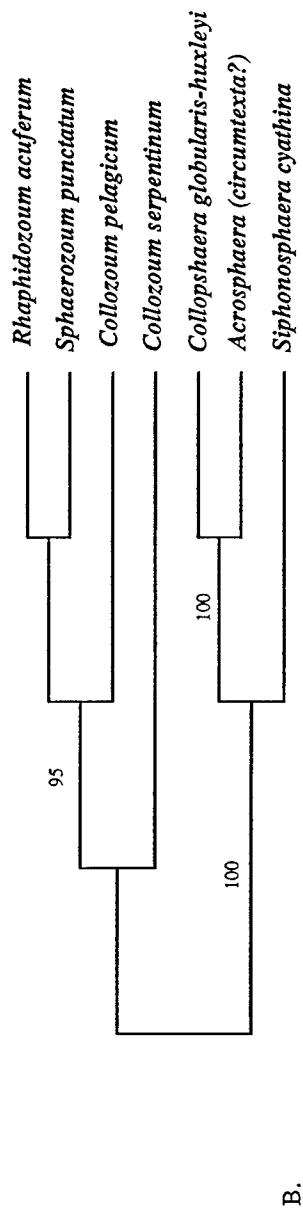
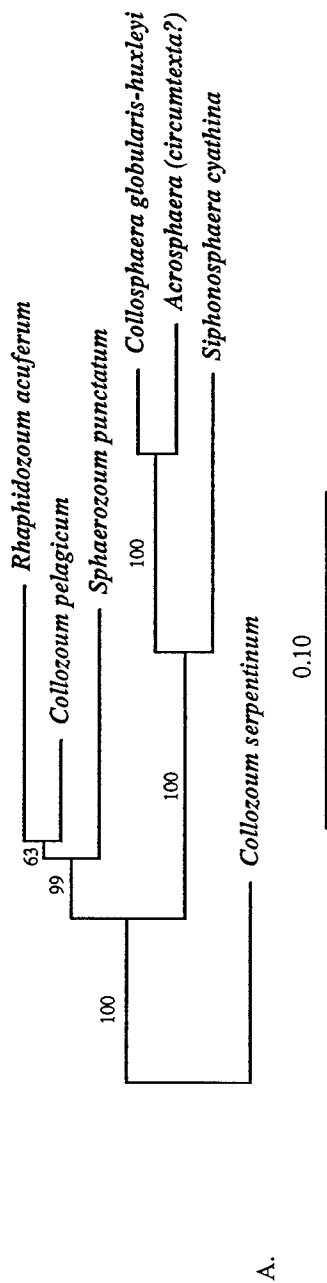
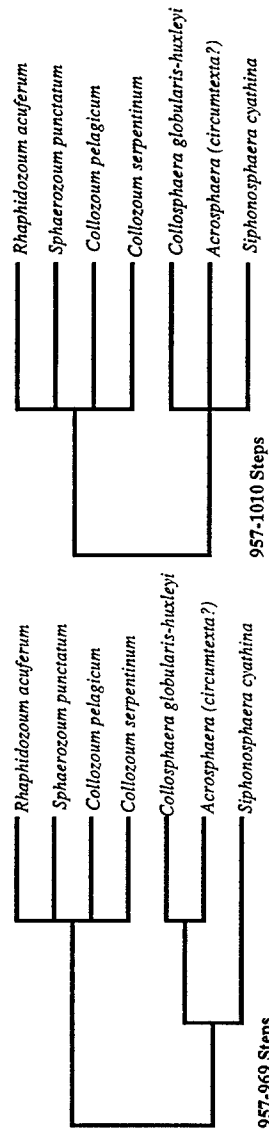
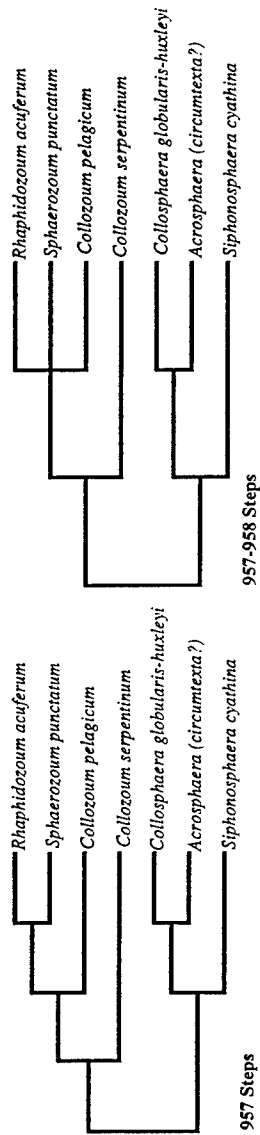


Fig. 5. Results from a decay analysis of the most parsimonious tree obtained from an exhaustive search restricting the taxa to just the colonial spumellaria. The number of additional steps required to produce the consensus trees with progressive degrees of collapse of major nodes is shown to the bottom left of each corresponding tree.



The support for branching order within the Sphaerozoidae also varied depending on the method of phylogenetic inference. In general, the branching patterns within the Sphaerozoidae were poorly resolved in the parsimony and maximum likelihood consensus trees (not shown). However, there was weak support for the grouping of R. acuferum, C. pelagicum and S. punctatum in the parsimony consensus tree (bootstrap value of 51%). The highest bootstrap support values were obtained from distance analyses and supported a branching order which separated the two species of Collozoum, placing one species branching early in the Sphaerozoidae and another sharing common ancestry with spicule-bearing genus R. acuferum. This tree also separated the two more commonly occurring, spicule-bearing species S. punctatum and R. acuferum.

The results from "unrooted networks" (Fig. 4, 5) provided limited additional information on the branching patterns within the Sphaerozoidae. Although the branching order was not better resolved by restricting the analysis to just the colonial spumellaria, better branching support emerged from the maximum likelihood analysis, which agreed with parsimony analysis, and placed the R. acuferum together with S. punctatum and C. pelagicum (bootstrap value of 95%).

## DISCUSSION

Phylogenetic reconstructions based on SSU rRNA coding regions challenge existing theories regarding the evolutionary history of the colonial spumellarian radiolaria. The data from molecular phylogenetic analyses indicate that the ability to form colonies may have evolved more than once in the evolution of the spumellarian radiolaria. The distance matrix method produced a tree topology which could not resolve the branching order of T. nucleata, a solitary spumellarian, relative to the two families of colonial spumellaria examined. The maximum likelihood tree did not show strong support for the branching order of T. nucleata relative to the two colonial families. Only a bootstrap value of 61% obtained in the maximum parsimony analysis alone separated the colonial spumellaria from



T. nucleata. Furthermore, the weak support for the monophyly of the colonial spumellaria was identified in a decay analysis in which the most parsimonious tree collapsed the node separating T. nucleata from the representatives of the Sphaerzoidae after only 3 steps. All of the above indicate that the node separating the colonial spumellaria from the solitary T. nucleata is not very robust.

The geological records of the colonial spumellaria have been used to yield information on their evolution. Based on observations from the fossil record, Bjørklund and Goll (1979) have suggested that coloniality may have evolved independently in the Collosphaeridae and the Sphaerzoidae. These authors argued that there is no evidence for the common ancestry of the Collosphaeridae and Sphaerzoidae in the fossil record. They state that the first occurrence of Sphaerzoidae in the fossil record is much earlier (Lower Oligocene) than the Collosphaeridae (basal Miocene) and that the distributions of the Sphaerzoidae are typically high-latitude whereas the Collosphaeridae originated and diversified from equatorial regions. More importantly, these authors assert that because the first occurrences of the Collosphaeridae in the fossil record are abrupt and characterized by fully formed lattice shells, it is probable that latticed shells were not the result of fusion of the spicules.

The conclusions made by the above authors based on the fossil record, however, appear to be difficult to test rigorously. Since skeleton-forming colonial spumellaria are not preserved in their colonial form in the fossil record, it is impossible to know which fossil forms actually produced colonies. In fact, this very problem lead Haeckel to give different species names to some shell-bearing fossil forms which were later found to be synonyms of colony-forming spumellaria. Furthermore, the existence of solitary-stages of Sphaerzoidae and the occurrence of spicule-bearing spumellaria which have never been observed to form colonies brings into question the phylogenetic importance of colony formation. For example it has been suggested that the genus Collozoum may have

members which all have solitary stages (Swanberg, 1979). Given the relative phylogenetic positions obtained in the distance analysis for the two Collozoum species, both of which have been cited as having solitary stages, we might conclude that coloniality may not be a definitive phylogenetic character. Likewise, while T. nucleata has never been observed to form colonies, we have to consider the possibility that its exclusively solitary habit may be a secondarily derived characteristic. If this is the case, similar arguments could be used for the existence of other exclusively solitary spumellaria so it is unclear that this question can be easily resolved even with additional sequence data from solitary forms.

The absence of solitary forms in the Collosphaeridae, however, is noteworthy. Strelkov and Reshetnyak (1971) hypothesized that the skeleton of the Collosphaeridae is derived from an ancestor with spines which merged to form a skeletal structure. A similar perspective on the possible phylogenetic relationships of the colonial spumellaria was reached by Anderson and Swanberg (1981) in their analysis of skeletal morphogenesis in representatives from the Collosphaeridae. These authors proposed a mechanism for shell deposition in colonial spumellaria which involved the precursory production of "cytokalymma" (differentiated extracapsular cytoplasm), followed by deposition of "organic nucleation centers" which serve as the matrix for the developing silicate shell. The authors described two methods of shell morphogenesis (bridge-growth and rim-growth) which they submitted could account for the variations in pore characteristics and shell ornamentation such as spines and tubules. Like Strelkov and Reshetnyak, these authors suggested that shell-bearing forms evolved from a spicule-bearing ancestor and that lattice shells are the result of the fusion of bar-like elements.

The phylogenetic reconstructions carried out in this study unanimously supported the monophyly of shell-bearing colonial spumellaria belonging to the family Collosphaeridae. Strong support was identified by both high bootstrap values (100% in all cases) and robust Bremer (decay analysis) support. Branching patterns within the Collosphaeridae indicate

that Siphonosphaera diverged prior to the split of Collosphaera and Acrosphaera. Evidence from physiological and electron microscopy studies indicates that the tubelike-projections seen in members of the genus Siphonosphaera may be the result of silicification after cytoplasmic streaming which is exhibited by all members of the spumellaria, as well as many other protista (Cachon and Cachon 1972; Anderson and Swanberg 1981; Anderson 1981). While the tube-like projections displayed by S. cyathina are very symmetrical, other species of the genus possess tubular ornamentation which is irregular and bears a striking resemblance to cytoplasmic shapes created during cytoplasmic streaming (Anderson and Swanberg 1981).

The observed divergence of Siphonosphaera prior to Collosphaera and Acrosphaera is contrary to an hypothesis presented by Strelkov and Reshetnyak (1971). These authors speculated that members of the genus Collosphaera represent a more primitive line of decent and that Acrosphaera and Siphonosphaera represent more derived forms. They argued that the smooth latticed skeletons possessed by the members of the genus Collosphaera represent more primitive features than the more elaborate skeletons of the genus Acrosphaera, which have a spiny appearance or those of Siphonosphaera which possess tube-like projections. Anderson and Swanberg (1981) also stated that spines and tubule ornamentation are most likely more derived features. However, if cytoplasmic streaming is fundamental in the formation of the tube-like projections possessed by Siphonosphaera, one can imagine that these structures may have arisen any time in evolution and possibly even more than once.

The monophyly of the Sphaerozoidae was well-supported in the distance analysis (bootstrap value of 88% leading to this family) however parsimony and maximum likelihood methods generated tree topologies which were in general poorly supported by the bootstrapping method.. A well-supported branching pattern was also identified within the Sphaerozoidae using distance methods. The branching pattern for the distance analysis

separated the two Collozoum species indicating a separate ancestry for the two Collozoum taxa. This pattern indicates that a secondary loss of skeletal material (i.e. spicules) occurred within C. pelagicum.

A similar conclusion about secondary skeletal loss was reached by Strelkov and Reshetnyak (1971). These authors proposed that the absence of skeletal elements is a secondary phenomenon and that the common ancestor of the Sphaerzoidae was spicule-bearing. They apparently attributed the secondary loss of skeletal elements to the fact that members of genus Collozoum, which are typically free of any skeletal material, are very infrequently found to possess simple spicules in their cytoplasm. These authors fail to consider that the occurrence of these spicules may be due to ingestion of other spumellaria or other spicule-bearing protists (Anderson, personal communication). Therefore, absence of skeletal features (which largely defines the genus Collozoum) may not be a reliable phylogenetic marker.

Collozoum serpentinum differs most noticeably from C. pelagicum by the characteristics of its central capsule. The central capsule in C. serpentinum is elongated and often forms twisted loops whereas in C. pelagicum it is characterized by digitform apophyses which are often branching at the ends. Interestingly, in maximum parsimony and maximum likelihood analyses of the "unrooted" network phylogenies, C. pelagicum was observed to branch with R. acuferum and S. punctatum, both spicule-bearing colonials. Although perhaps only coincidental, the shape of the apophyses on the central capsule of C. pelagicum bears a crude resemblance to the spicules of R. acuferum and S. punctatum suggesting a possible evolutionary connection between these apophyses and the radiate spicules possessed by Rhaphidozoum and Sphaerzoum.

Based on our molecular results, the diversity within the Spumellarida, both solitary and colonial forms, should not be understated. Molecular phylogenetic analyses of the SSU rRNA genes in this study revealed diversity within the colonial and solitary spumellaria that

rivals that observed in many other protist groups analyzed to date. An explanation for such divergence within this order is wanting given our incomplete understanding of generation times and other factors which would affect the rate at which these protists evolved.

Other questions remain concerning the degree of variability within an individual colony. In a recent study, Petrushevskaya and Swanberg (1990) examined the morphological variability in the Collosphaeridae. These authors concluded that much of the variability seen within a colony is due to environmental differences and that sexual reproduction is probably absent in colonial radiolaria. However, geologists have reported "hybridizations" in Collosphaeridae which they believe are attributable to sexual reproduction (Bjørklund and Goll 1979). The question of whether or not colonial spumellarians are indeed entirely clonal can now be examined by comparing the SSU rRNA genes from several individuals from the same colony.

While this molecular study has not resolved of the issue of the evolution of coloniality among the spumellaria, it has raised the question of the importance of this character in determining relationships among the Spumellarida. This analysis, while far from a complete molecular diagnosis, has revealed potential avenues for further exploration into colonial spumellarian evolution. We believe that the molecular tools designed during this work will be helpful in determining the extent to which morphological variability seen in colonial spumellarians is reflected at the genetic level. Such studies as this will hopefully provide much-needed insights into the life history of these morphologically and genetically diverse protists.

#### ACKNOWLEDGMENTS

This material is based upon work supported under a National Science Foundation Minority Graduate Fellowship. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation. Further financial support is acknowledged in part

from NSF (OCE-9314533) (to D. A. Caron) and from the Scurlock Fund for research at the Bermuda Biological Station for Research, Inc. (BBSR), the Grant-In-Aid Fund of BBSR, funds from the Woods Hole Coastal Research Center, the Education Office of the Woods Hole Oceanographic Institution and the Lerner Gray Fund for Marine Research of the American Museum of Natural History (to L. A. Amaral Zettler). We thank Dr. Rebecca Gast for critically reading the manuscript. We thank Drs. O. Roger Anderson and Neil Swanberg for help in identifying the species of colonial spumellaria used in this work. We wish to thank Dr. Anthony Michaels of the Bermuda Biological Station for help with identifications of specimens. The authors also wish to further acknowledge the assistance of Dr. Anthony Michaels for opening up his lab and allowing lab members especially Frances Howes and numerous other volunteers to assist with blue water diving collection of specimens.

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## **Chapter 4**

### **Phylogenetic Relationships among Three Orders of Acantharea Based on SSU rRNA Gene Sequencing**



## SUMMARY

The phylogeny of the Acantharea was examined using small-subunit ribosomal RNA (SSU rRNA) gene sequence analysis of two previously sequenced (Chapter 1) acantharia along with additional representatives from the Symphyacanthida, Chaunacanthida and the Arthracanthida. Our previous studies showed that Acantharea form a monophyletic group branching as an independent protist lineage among crown groups but not directly related to any of them. The results from this more in-depth molecular analysis of the branching patterns within the Acantharea revealed a phylogeny which is not entirely consistent with morphology-based phylogenies. In particular, the phylogenetic placement of Haliommatidium sp. was in disagreement with its current taxonomic placement among the Symphyacanthida. In molecular analyses described herein, Haliommatidium clustered with members of the order Arthracanthida. Apart from cyst formation and number of axopodial exit pores, Haliommatidium sp. shares several morphological features with the Arthracanthida which support these molecular results.

Key words: Acantharea, evolution, molecular phylogeny, small-subunit ribosomal RNA

## INTRODUCTION

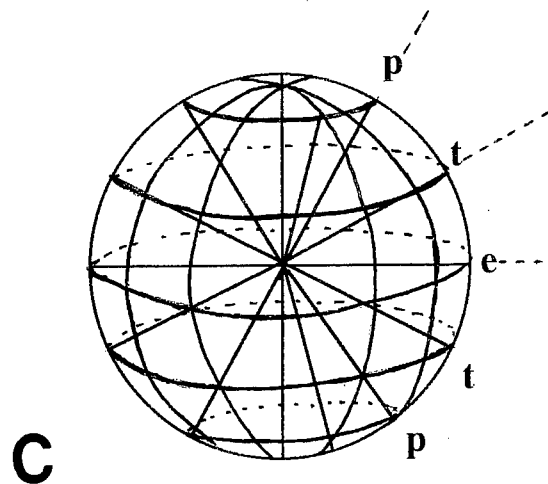
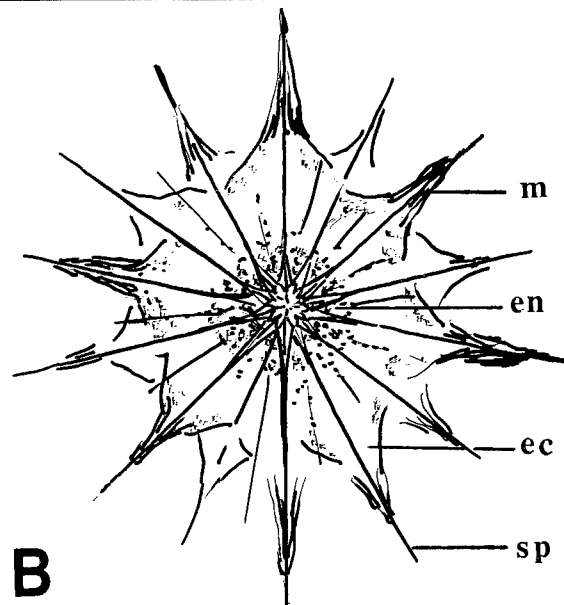
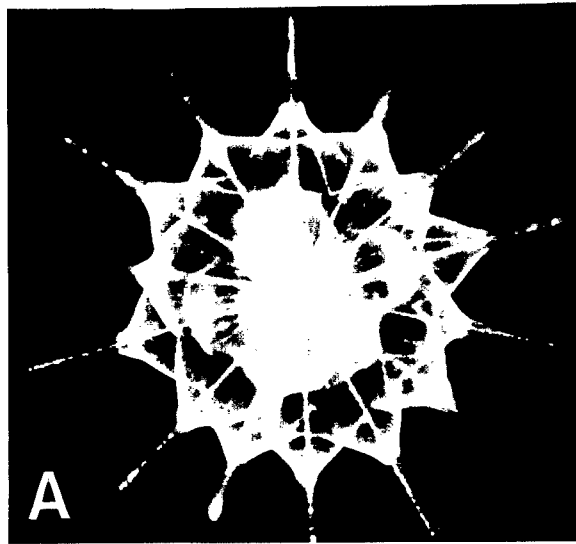
Members of the class Acantharea are heterotrophic planktonic sarcodines which are common components of open ocean environments. In addition to their role as consumers, acantharia also contribute to primary productivity in the ocean via their symbioses with eukaryotic algae. Acantharia typically occur at densities of 10 cells  $l^{-1}$  (Caron and Swanberg 1990), however, they have occasionally been found to dominate the biomass of microzooplankton during "bloom-like" conditions (Merinfeld 1969; Massera Bottazzi and Andreoli 1981; Febvre 1990) where densities from 30-35 cells  $l^{-1}$  have been reported (Michaels 1988). While acantharian abundances in the world oceans have been underestimated in the past improved methods of sampling and preservation (Michaels 1988) are revealing their abundances in the plankton.

Acantharian cells are divided into the endoplasm and the ectoplasm (see Fig. 1 A, B), which are separated by a capsular wall in one order of acantharia, the Arthracanthida. The ectoplasm is encompassed by the periplasmic cortex or outer pellicle, and also the outermost layer, the calymma. The calymma houses the characteristic acantharian non-actin-filaments called myonemes. The myonemes are contractile bundles located around the tips of the skeletal spines and are postulated to contribute to active vertical motion in the acantharia (Febvre 1981; Reshetnyak 1981; Febvre and Febvre-Chevalier 1982).

Acantharia are further distinguished from other protists on the basis of the Müllerian-arrangement of spines and their skeletal composition. The acantharian skeleton is organized in a highly symmetrical fashion known as Müller's law, in which 10 diametric or 20 radial spines come together at the center of the cell to form a characteristic geometric pattern (see Fig. 1C). The acantharia are the only protists known to construct skeletons of monocrystals of strontium sulfate (Schröder 1907; Hollande and Cachon-Enjumet 1963; Massera-Bottazzi and Vinci 1965), although other protistan groups are known to metabolize strontium sulfate or similar alkaline earth compounds (Fenchel and Finlay 1986;



Fig. 1. **A.** An unidentified acantharian specimen. **B.** Schematic diagram showing the location of the myonemes (m), endoplasm (en), ectoplasm (ec) and spines (sp). **C.** Müllerian arrangement of spines (after Febvre, 1990). (p) polar spine; (t) tropical spine; (e) equatorial spine.







Raven et al. 1986). As such, acantharia play a role in the cycling of strontium in the ocean (Bernstein et al. 1987) and some attempts have been made to use the levels of  $\text{Sr}^{90}$  incorporated into acantharian skeletons as a means of measuring radioactivity in the oceans (Schreiber and Ortalli 1964; Strelkov and Reshetnyak 1974).

Like other skeleton-bearing sarcodines, the acantharia were first classified on the basis of their skeletal morphology (Müller 1858; Hertwig 1879; Haeckel 1888). These authors all considered Acantharia as members of the "Radiolaria", a now defunct formal taxonomic term whose original definition encompassed them (see Chapter 1 for a more in-depth discussion of the differences between Acantharea and Polycystinea). Schewiakoff (1926) is credited with establishing a classification scheme which incorporated aspects of acantharian cytology and skeletal morphology. His 1926 monograph first recognized the Acantharia as distinct from the "radiolaria" (Polycystinea and Phaeodarea) and still serves as the foundation of modern-day classifications. Despite the need for systematic revision noted in the latest protistology reviews of the acantharia (Cachon and Cachon 1985; Febvre 1990) the past decade has seen very little systematic revision within the Acantharea. The latest treatments of the group include Trebougoff (1953) and Reshetnyak (1981) (in Russian).

There are 150 species, 50 genera, 20 families and 4 orders of acantharia reported in the most recent literature (Febvre 1990). Morphology-based systematic work requires the labor-intensive and time-consuming techniques of treatment of specimens with sulfuric acid prior to observation under the light microscope or use of electron-microscopy to determine species-level identifications. Furthermore, the phylogenetic significance of some of the criteria used in distinguishing between different taxa (such as nature of the central juncture of the spines (after treatment with sulfuric acid)) have not been challenged. Since the acantharia lack a fossil record, there are few alternative methods available for comparing how well existing systematic schemes reflect phylogenetic relationships.

Ribosomal RNA-based phylogenetic approaches offer an alternative means of inferring relationships within the Acantharea. Recent cloning and sequencing efforts of small-subunit ribosomal RNA genes (Chapter 1) show a branching of Acantharea among crown groups. In this paper, we examine more closely the branching pattern of three orders of Acantharia in an effort to compare existing taxonomic frameworks with the results from this study.

## MATERIALS AND METHODS

One very practical problem with the methods used in making accurate identifications of acantharia is that they typically destroy cytoplasmic material in the process. This makes microscopic identification at the light-microscope level difficult and in some cases only allows for order or family-level identifications with confidence. However, in certain groups, especially within the Arthracanthida, as well as, the Symphyacanthida (such as Haliommatidium), there are representatives which can be identified live to genus-level due to very distinctive features.

Individuals were given sample numbers prior to identification. In this study, all identifications were made by Dr. A. F. Michaels (Bermuda Biological Station for Research, Inc., Bermuda) who is a specialist in acantharian biology. Acantharian samples used in this paper were: Arthracanthid 205 (Order: Arthracanthida, Suborder: Sphaenacanthina, Family: Acanthometridae, Acanthometra sp.), Arthracanthid 206, (Order: Arthracanthida), Symphyacanthid 211 (Order: Symphyacanthida), Chaunacanthid 217 (Order: Chaunacanthida), and Chaunacanthid 218 (Order: Chaunacanthida), and Symphyacanthid 235 (Order: Symphyacanthida, Family: Pseudolithidae, Haliommatidium sp.).

All specimens were collected in glass or polycarbonate jars by divers off the southwestern coast of Bermuda in September 1994. Specimens were maintained in 0.22  $\mu$ m Millipore-filtered Sargasso Sea water in glass culture tubes with brine shrimp (Artemia salina) as food until sacrificed for molecular analysis. Whenever possible, reproductive

acantharia, which are often characterized by cyst-formation, were sacrificed for molecular analyses. The rationale for using reproductive individuals was to obtain samples that were highly enriched with sarcodine DNA over non-target DNA's such as prey or symbiotic algal DNA which may be present in the sample.

Individual central capsules or cysts were passed through several 0.22  $\mu$ m-Millipore filtered seawater rinses followed by a final MilliQ (distilled, deionized)-water rinse. Specimens were then placed in a modified 1X PCR buffer solution which consisted of 50 mM KCl, 10 mM Tris, pH 8.3, 2 mM MgCl<sub>2</sub>, 0.001% Gelatin, and 1.0% NP40 (Sigma; St. Louis, MO). Cells were then stored frozen at either -20°C or -70°C. Samples for molecular analyses were heated at 95°C for 10 minutes to lyse cells and liberate DNA. An aliquot of the lysed sample was used directly in PCR amplification reactions (Saiki et al. 1988). Typically anywhere between 1 and 5  $\mu$ l of a 20 $\mu$ l sample lysed in this manner yielded strong PCR amplifications.

Arthracanthid 206, Chaunacanthid 217 and Symphyacanthid 235 (*Haliommatidium* sp.) 16S-like rDNAs were amplified using PCR and eukaryotic primers specific to the ends of the molecule (Medlin, 1988) and subsequently cloned into a double-stranded TA plasmid vector pCRII (Invitrogen; San Diego, CA). Plasmid DNA was purified using Promega Wizard Midiprep (Promega; Madison, WI) kit and methods. Remaining samples, Arthracanthid 205 (*Acanthometra* sp.), Symphyacanthid 211, and Chaunacanthid 218 SSU rRNA genes were PCR-amplified in two overlapping fragments using one acantharian-specified primer in combination with either the forward or reverse Medlin primer to yield a final full length product. These acantharian-specific primers were synthesized as described in Chapter 1 and consisted of the forward primer A497, 5'-GTGAGTTGATTGGAATGA-3' and the reverse primer A899, 5'-TCGTCATACAAAGGTCCA-3'.

All PCR fragments were purified using the Wizard PCR Prep system (Promega; Madison, WI). Direct sequencing of PCR products as well as cloned plasmid DNA was

accomplished using reagents from the SequiTherm Long Read Sequencing Kit (Epicentre Technologies; Madison, WI) along with the SequiTherm Cycle sequencing protocol developed by Li-Cor which consisted of 5 minutes of denaturation at 95°C prior to 30 cycles of 20 sec at 95°C (30 sec for plasmid DNA), 30 sec at 60°C, and 1 minute at 70°C using a Perkin Elmer 2400 Thermo Cycler. Sequenced templates were run out on a Licor model 4000L sequencing machine.

Gel images were transferred from Licor to BioImage (Millipore Corp; Ann Arbor, MI) and sequences were analyzed using the BioImage DNA Sequence Film Reader software. The 16S-like rRNA sequences of acantharian samples were aligned against a subset of the total eukaryotic alignment data base (Olsen et al. 1992). Sequences were aligned by eye using the Olsen Multiple Sequence Alignment Editing program with regard to primary and secondary structural conservation. Phylogenetic analyses employed distance (Olsen 1988), maximum parsimony (Swofford 1991) and maximum likelihood (Olsen et al. 1994) methods. The sites used in this analysis included 1,368 positions and was identical to the one used in the analyses to infer the phylogenetic placement of acantharia relative to the polycystine radiolaria (Chapter 1). The 1 base pair difference (e.g. 1,369 positions used in Chapter 1 compared with 1,368 positions used in this study) is due to one site becoming a gap when the data set was restricted to the acantharian and two polycystine spumellarian sequences. Thalassicolla nucleata and Collosphaera globularis-huxleyi were used as outgroups in the analyses. Bootstrap (Felsenstein 1985) and decay (Bremer 1988) analyses were conducted to provide a means of relative branch support.

## RESULTS

All phylogenetic reconstructions accomplished yielded identical tree topologies. However, bootstrap values obtained for the three methods differed and are indicated on the nodes of the consensus parsimony tree shown in Fig. 2. Distance bootstrap values are listed on top, parsimony in the middle, and maximum likelihood on the bottom. In general,

all analyses favored the branching of the Symphyacanthida with the Chaunacanthida and segregated these two orders from the Arthracanthida. Haliommatidium sp., currently classified as a symphyacanthid, was observed to branch with the Arthracanthida in all analyses. Haliommatidium sp. branched with Acanthometra sp. with moderate support in all analyses but the distance analysis, wherein the branch order between Acanthometra sp., Haliommatidium sp. and Arthracanthid 206 was poorly resolved.

In addition to bootstrapping, the stability of branching was tested further in a decay analysis depicted in Fig. 3. The decay analysis was accomplished by first performing an exhaustive search using PAUP 3.1.1 to obtain the length of the most parsimonious tree, and then sequentially adding steps to the value of the shortest tree found using the initial upper bound setting of the branch and bound search option. Resulting trees constructed at each additional step-allowance were then consensed in a strict consensus tree, and the order in which various clades "decayed" was compared.

The trees depicted in Fig. 3 show the single most parsimonious tree obtained which was 608 steps long, followed by the strict-consensus trees from 609, 614, 615 and 621 steps respectively. After 1 additional step (608-609), the node joining Haliommatidium sp. and Acanthometra sp. collapsed. Six steps (608-614) were required for the collapse of the chaunacanthid clade. After 7 steps (608-615) the connection between the chaunacanthid clade and Symphyacanthid 211 was lost. Complete loss of structure in the acantharian lineage resulted after 13 steps (608-621) with the collapse of the "arthracanthid" clade (including Haliommatidium sp.).

Fig. 2. The most parsimonious tree inferred from of an exhaustive search using maximum parsimony. Distance and maximum likelihood analyses yielded the same tree topology. Three sets of bootstrap values are given for each method as follows: distance (top value), maximum parsimony (middle value), maximum likelihood (bottom value). Only values greater than 50% are shown, the dash for the node leading to Arthracanthid 205 and Symphyacanthid 235 indicates a distance bootstrap value which was less than 50%. Acantharian sequences are indicated in bold.

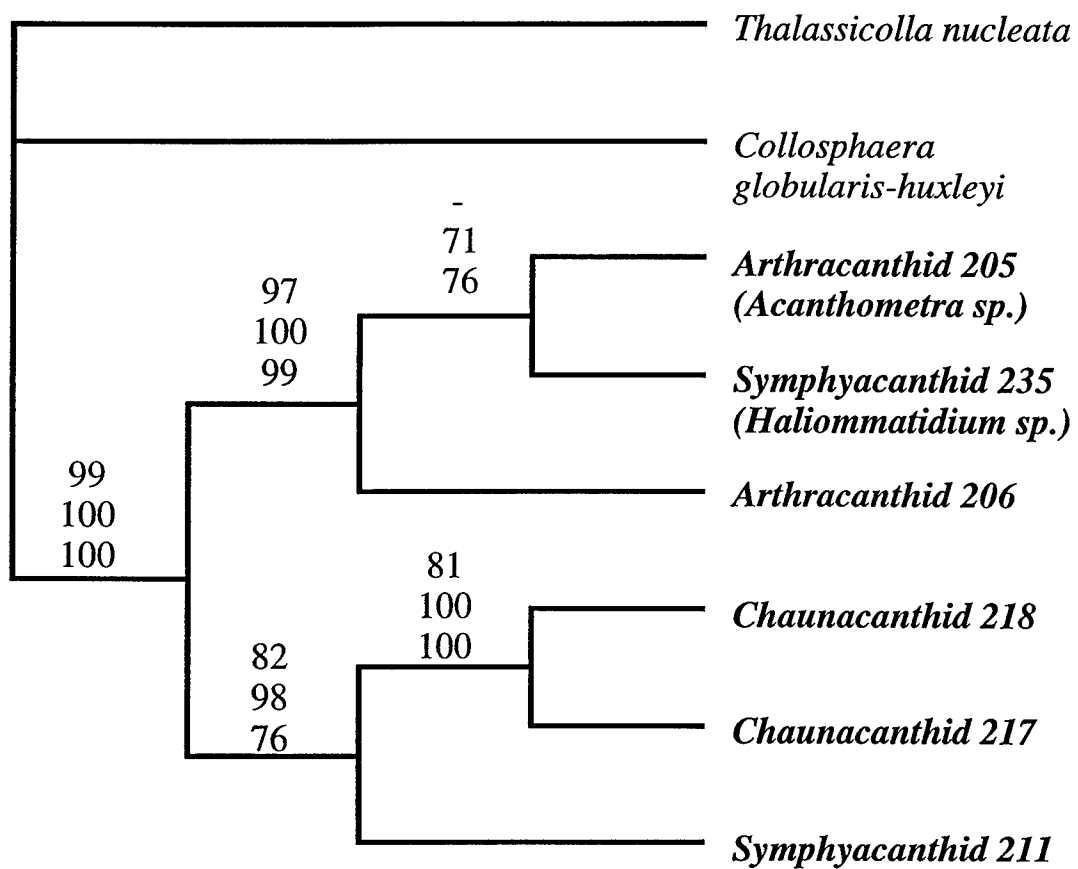
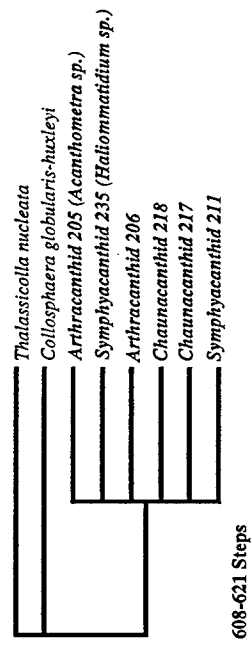
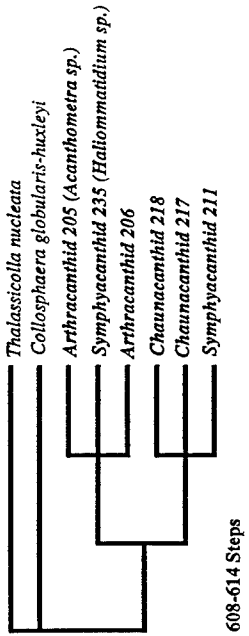
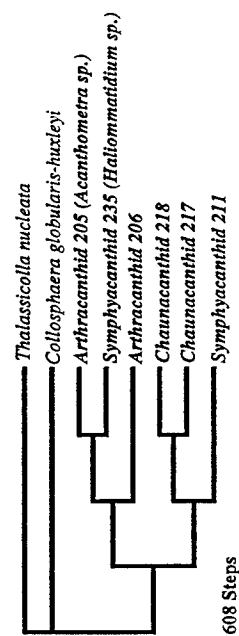
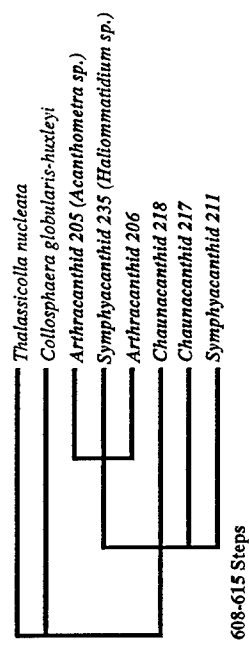
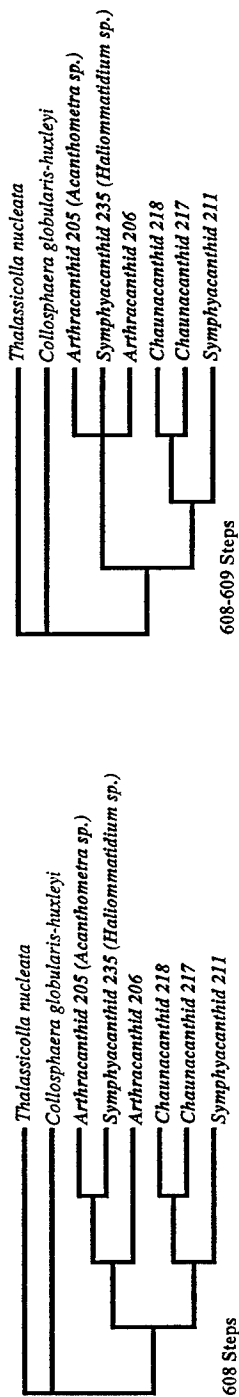


Fig. 3. The results of a decay analysis conducted using the most parsimonious tree from an exhaustive PAUP search. Strict consensus trees obtained after 1 step (608-609), 6 steps (608-614), 7 steps (608-615), and 13 steps (608-621) indicate the order of decay of the major clades in the acantharian lineage. Acantharian sequences are represented in bold.





## DISCUSSION

The current classification scheme for the Acantharea, based on morphological criteria established by Schewiakoff in his 1926 monograph, has been accepted with little formal systematic revision since that time (Reshetnyak 1981; Febvre 1990). Some specialists acknowledge that members of the symphyacanthid family Pseudolithidae, of which Haliommatidium is a member, require taxonomic reevaluation (Cachon and Cachon 1982; Cachon and Cachon 1985) but no formal revisions have been suggested to date. The results from this study found the placement of members of the Arthracanthida and the Chaunacanthida to be consistent with the systematic scheme proposed by the above authors. However, the results for the Symphyacanthida indicate that some of the morphological criteria used in defining the symphyacanthid clade are not reliable phylogenetic markers.

One of the major results of this study was the branching of Symphyacanthid 235 (Haliommatidium sp.) with Arthracanthid 205 (Acanthometra sp.) and an unidentified arthracanthid, Arthracanthid 206 within the arthracanthid clade. This result was well-supported by both the bootstrapping and decay analysis results. Reexamination of the literature available on the morphology and cytology of Haliommatidium, however, reveals some salient features shared by Haliommatidium and members of the Arthracanthida which substantiate this result.

The features which distinguish members of the Arthracanthida from other orders of acantharia include the following: the existence of a central capsule; a well-defined body plan possessing latticed or armored shells; the presence of a small number of apertures in the calymma for the axopodia to exit, and an increase in the number of myonemes compared to other orders (from 24-40). In considering these criteria, there are several morphological features of Haliommatidium which might place it among the Arthracanthida.

When we compare the above features to those found in Haliommatidium we see that

Febvre (1990) makes note of a very conspicuous central capsular wall in Haliommatidium as is seen in most Arthracanthida. In addition, Haliommatidium forms a latticed shell through the fusion of the apophyses on its spines, similar to those that can be seen in members of the Arthracanthida. Furthermore, Haliommatidium is known to possess 23-34 myonemes as compared with the 8-12 myonemes possessed by other members of the order Symphyacanthida (Strelkov and Reshetnyak 1974).

One difference between Haliommatidium and members of the Arthracanthida lies in the number of apertures for the exit of axonemes which number between 30-40 in the family Pseudolithidae whereas there are many fewer in the Arthracanthida. Another difference between Haliommatidium and members of the Arthracanthida is that Haliommatidium forms a cyst prior to swarmer formation, whereas no members of the Arthracanthida form cysts. The cysts formed by Haliommatidium, however, develop differently than those of other cyst-forming Symphyacanthida such as members of the Astrolithiidae.

Other differences exist between Haliommatidium and other Symphyacanthida members. For example, one of the distinguishing features of the Symphyacanthida is the inability of the central skeletal mass to be dissociated with sulfuric acid treatment. The central body of members of the genus Haliommatidium can be dissociated by sulfuric acid treatment whereas dissociation does not occur in most other Symphyacanthida. Finally, another striking difference found in Haliommatidium that is not seen in any other acantharian let alone symphyacanthid, is a single large nucleus during the trophic stage of the organism instead of the many nuclei observed in all other types of vegetative acantharia. The evolutionary significance of this mononuclear condition seen in Haliommatidium remains enigmatic. In any event, many morphological features possessed by Haliommatidium set it apart from other symphyacanthids.

The branching of Symphyacanthid 211 relative to the chaunacanthid clade is also noteworthy. The data indicate that the Symphyacanthida diverged prior to the

Chaunacanthida. This hypothesis is contrary to what has been suggested based on the morphological data alone (Schewiakoff 1926; Strelkov and Reshetnyak 1974; Reshetnyak 1981). These authors suggest that based on myoneme number and skeletal complexity, the Symphyacanthida are probably more derived than the Chaunacanthida. However, given that the Symphyacanthida (as it is currently defined) was shown to be polyphyletic in this analysis, the branching order of the Symphyacanthida relative to the Chaunacanthida is best determined only after the analysis of additional symphyacanthid sequence data.

The results from this study revealed that analysis of SSU rRNA genes may prove useful in future taxonomic revision within the Acantharea, at least at the order level of taxonomic organization. The additional acantharian order the Plegmacanthida (Reshetnyak 1981) (not yet formally recognized) along with representatives from the Holacanthida and representatives of a once proposed fifth order, the Actineliida (Levine et al. 1980) await molecular investigation.

#### ACKNOWLEDGMENTS

This material is based upon work supported under a National Science Foundation Minority Graduate Fellowship. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation. Further financial support is acknowledged in part from NSF (OCE-9314533) (to D. A. Caron) and from the Scurlock Fund for research at the Bermuda Biological Station for Research, Inc. (BBSR), the Grant-In-Aid Fund of BBSR, funds from the Woods Hole Coastal Research Center, the Education Office of the Woods Hole Oceanographic Institution and the Lerner Gray Fund for Marine Research of the American Museum of Natural History (to L. A. Amaral Zettler). We thank Drs. Annika Sanfilippo and William Riedel for offering reprints of their translations of Russian references in their collection, as well as making available other hard-to-find references. We also thank Dr. Alexi Shalapyonok for assistance with translating Russian manuscripts and

Gaspar Taroncher for assistance in German translation. We wish to thank Dr. Anthony Michaels of the Bermuda Biological Station for help with identifications of specimens. The authors also wish to further acknowledge the assistance of Dr. Anthony Michaels for opening up his lab and allowing lab members especially Frances Howes and numerous other volunteers to assist with blue water diving collection of specimens.

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## **Chapter 5**

### **Conclusions**



The work described in this thesis is significant for its contribution to eukaryotic molecular phylogeny and to the field of acantharian and polycystine biology. The sequence data obtained are the first SSU rRNA genes to be obtained from the Acantharea and Polycystinea respectively. They represent the first molecular genetic work to be accomplished on representatives of either of these two classes. The writing of this thesis and subsequent publication of the contents of its chapters as manuscripts in scientific journals will hopefully instigate further applications of molecular approaches to answering questions in the biology of these understudied protists.

The most significant result from this thesis comes with the finding that based on SSU rRNA gene-based phylogenies, the Acantharea and the Polycystinea do not share common ancestry (Chapter 1). These results imply that the taxon Actinopoda (as well as any other taxon uniting Acantharea and Polycystinea such as Cavalier-Smith's newly proposed "Radiozoa") is not monophyletic and should be discarded in future systematic revisions of the Sarcodina. These results are actually in agreement with speculations made by a variety of authors who have submitted that axopodia are convergent structures which are probably the result of ecological constraints placed on organisms possessing a common planktonic mode of existence (Cachon and Balamuth 1977; Merinfeld 1978; Shulman and Reshetnyak 1980; Merinfeld 1981; Reshetnyak 1981). The results from Chapter 1 also support the monophyly of the Acantharea and at least the separate monophyly of the order Spumellarida of the Polycystinea.

The extent to which SSU rRNA genes differ within a given species of Thalassicolla nucleata was the focus of Chapter 2. The amount of variation observed within a species of this genus collected from the Sargasso Sea and the Pacific Ocean was higher than one might expect for a single species, with the highest values falling at levels observed at the genus level in other taxa. Perhaps given the amount of divergence displayed within the

spumellarian SSU rRNA genes sequenced during this thesis, these values should not be surprising.

Whether this degree of genetic variation warrants new species or strain designations in the Thalassicolla genus is unclear but raises the important question of what defines a species in Thalassicolla. The existing species designations made by Haeckel for Thalassicolla are suspect because they occur so infrequently in the literature following their initial descriptions, and also because they include a total of seven species, four of which are lacking in symbionts. Given our present understanding of the feeding behavior within Thalassicolla and the importance of symbiont-derived nutrition, it seems that some of these species may not be valid. These facts in combination with morphological changes associated with parasitism, differences in physiological condition, and external factors such as excessive wave agitation, may have contributed to the morphological features used by Haeckel to describe different species of this genus.

The third chapter examined the phylogeny of the colonial spumellaria and attempted to determine whether or not the colonial radiolaria represent a monophyletic evolutionary assemblage. One robust conclusion drawn from this chapter was the monophyly of the Collosphaeridae, which is comprised of shell-bearing colonial forms. Representatives of three genera from this family grouped together with bootstrap values of 100% in all analyses accomplished. These results are exciting because they suggest a potential for determining further relationships between the Collosphaeridae and comparing them to phylogenies derived from the polycystine fossil record. These results also suggest a potential for establishing a phylogeny based classification for the Collosphaeridae.

Representatives of the two families of Spumellarida known to form colonies used in these analyses indicated that the colonial spumellaria may not be monophyletic. Because the different methods employed in reconstructing phylogenies did not yield the same answer, I cannot be fully confident of this result. The monophyly of the colonial

spumellaria was supported in only one of the methods (maximum parsimony) and with low bootstrap support (61%). Given these results, it appears that coloniality may not serve as a reliable phylogenetic marker.

All of these results come with the overwhelming revelation of the high sequence divergence exhibited by the Spumellarida. However, Hillis et al. (1996) discuss several possibilities that might account for the observed differences in heterogeneity rates seen within a given gene. Among them are differences in DNA repair efficiency and differences in exposure to mutagens, both of which may explain some of the source of this variability. It may be that the spumellaria, as planktonic organisms, are subjected to high levels of UV damage since they typically occur in the surface portions of water column. If spumellaria lack a means of protecting themselves from UV or else do not possess adequate DNA repair mechanisms to efficiently repair damaged DNA, this might explain some of the observed sequence divergence.

Furthermore, the low % G + C content found in the spumellarian sequences may make them more susceptible to thymine-dimer formations created during exposure to UV which may be difficult to repair with existing DNA Repair mechanisms. However, if UV radiation is acting as a selective force in the % G + C content of these organisms, we would expect to see high % G + C content not the low values observed thus far in the Spumellarida. This scenario has been proposed by Singer and Ames (1970) to account for the high % G + C content in bacteria inhabiting high UV-exposed environments. It seems equally likely that members of the Spumellarida may have evolved mechanisms to deal with UV and that the long branch lengths observed in the spumellarian phylogenies are attributable to other reasons such as long divergence times or fast organismal generation times.

The final chapter of this thesis examined the evolutionary relationships between three orders of Acantharea. The results from this work were consistent with the

morphology-based systematics in that they supported the monophyly of the Chaunacanthida and the Arthracanthida. The exception was in the phylogenetic placement of Haliommatidium sp. with the Arthracanthida. While this result is contrary to its current taxonomic position among the Symphyacanthida, it is less surprising when one reexamines the morphological features that Haliommatidium sp. shares with the Arthracanthida. Given this result, the formation of cysts (an ability possessed by Haliommatidium sp. but not members of the Arthracanthida) may not be a reliable phylogenetic marker whereas myoneme number and presence of a central capsule wall may be. In any event, it appears that the Acantharia are a more recently divergent lineage that are not closely related to any known protistan group for which there is currently SSU rRNA sequence information.

The molecular approach using an SSU rRNA-based method for reconstructing phylogenies of the Acantharea and the Polycystinea has proven to be a fruitful one. Within the spumellaria, especially, there appears to be sufficient sequence variation to make fine-scaled comparisons between existing morphospecies. The variability within the acantharian SSU rDNA was significantly less than that of the spumellaria, and may prove less useful in establishing differences at the species level. The design of acantharian and colonial spumellarian oligonucleotide probes and primers accomplished during this thesis, will assist in further efforts to establish a phylogeny-based systematic framework for both of these protistan groups. The application of the oligonucleotide probes also holds potential for addressing ecological questions surrounding the life cycle and distributions of these elusive protists.

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## **APPENDIX A: SEQUENCE ALIGNMENT**

.



"Sept4aln" on 5-SEP-96 15:33:41

1 (1369mask): 1.369 homologous sites included in phylogenetic analyses  
2 (MNELEIDY): L10826:Mniopsis leidy:Ctenophore  
3 (DIAGRAND): L10824:Diaphanoeca grandis:Choanoflagellate  
4 (ATHBOMBBA): M55638:Atella bombacina:fungus:basidiomycete  
5 (BLAEMERS): M54937:Blasotocladia emersonii  
6 (CHLREINH): M32703:Chlamydomonas reinhardtii:Chlorophyte:Volvocales  
7 (ORYSATIV): X00755:Oryza sativa (rice):Chlorophyte  
8 (PORASRUG): L27635:Porphyridium aeruginum:Rhodophyte:Bangiophycideae  
9 (ACACASTI): M13435:Acanthamoeba castellanii:'Amoebida'  
10 (PHRBALAM): L23799:Phreatamoeba balamuthi  
11 (STVALSID): L26204:Stylonema alsidii:Rhodophyte:Porphyridiaceae  
12 (EMTHUXLE): L04957:Emiliana huxleyi:Haptophyte  
13 (OCHDANIC): M32704:Ochromonas danica:stramenopile:chrysophyte  
14 (CAFROENB): L27633:Cafeteria roenbergensis:stramenopile:bicosoecid  
15 (LABMINTUT): L27634:Labyrinthuloides minuta:stramenopile:labyrinthulid  
16 (OXYGRANU): X53486:Oxytricha granulifera:ciliate:hypotrich  
17 (BLEAFERI): M95168:Hartmannella vermiformis:'Lobosa'  
18 (THEANNU): M84243:Theileria annulata:apicomplexa:Coccidia:piroplasm  
19 (SYNFILOI): M88518:Syndinium pilosum:dinoflagellate:zooxanthellales  
20 (ZBBSR205): Arthracanthid 205 (Acanthometra sp.)  
21 (ZBBSR206): Arthracanthid 206  
22 (ZBBSR235): Symphyacanthid 235 (Haliommatidium sp.)  
23 (ZBBSR218): Chaunacanthid 218  
24 (ZBBSR217): Chaunacanthid 217  
25 (ZBBSR211): Symphyacanthid 211  
26 (Pchroma): Paulinella chromatophora SSU rRNA, X81811  
27 (EUGROTUN): X77692:Euglypha rotunda CCAP 1520/1:Sarcodina  
28 (ENTOGING): Entamoeba gingivalis (st.ATCC30927)  
29 (DICTISCO): K02641:Dictyostelium discoideum:dictyostelids  
30 (Zthnucl): Thalassicola nucleata (BBS3) from the Sargasso Sea  
31 (W10279): Thalassicola "nucleata" clone number 79 collected from the Pacific sample W10  
32 (W10274): Thalassicola "nucleata" clone number 74 collected from the Pacific sample W10  
33 (W10272): Thalassicola "nucleata" clone number 72 collected from the Pacific sample W10  
34 (W10210): Thalassicola "nucleata" clone number 10 collected from the Pacific sample W10  
35 (ZBBSR7): Rhaphidozoum aciferum  
36 (ZBBSR173): Collosphaera globularis-huxleyi  
37 (Siphocyan): Siphonophora cyathina  
38 (ZBBSR2): Collozoum pelagicum  
39 (ZCR4): Sphaerozoum punctatum  
40 (ZCR16): Collozoum vermiformi  
41 (ZCR6A): Acrosphaera (circumtexta?)  
42 (PHYPOLYC): X13160:Physarum polycephalum:physarids  
43 (NAEGRUBE): M18732:Naegleria gruberi:schizopyrenids:vahlkampfiids  
44 (euk heli): possible secondary structure helices  
45 (rad):  
46 (rad):

| Position: | Sequence | Identity: |
|-----------|----------|-----------|
|-----------|----------|-----------|

162

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:    |
|----------------|-----------------------|----------|
| 104            | 1                     | 1369mask |
| 104            | 2                     | MNELEIDY |
| 104            | 3                     | DIAGRND  |
| 104            | 4                     | ATHOMBA  |
| 104            | 5                     | BLAEMERS |
| 104            | 6                     | CHLREINH |
| 104            | 7                     | ORYSATIV |
| 104            | 8                     | PORAERUG |
| 104            | 9                     | ACACAST1 |
| 104            | 10                    | PHRBALAM |
| 104            | 11                    | STYALSID |
| 104            | 12                    | EMTHUXLE |
| 104            | 13                    | OCHDANIC |
| 104            | 14                    | CAFROENB |
| 104            | 15                    | LAMBINUT |
| 104            | 16                    | OXYGRANU |
| 104            | 17                    | BLEAMERI |
| 104            | 18                    | HARVERMI |
| 104            | 19                    | THEANNUL |
| 104            | 20                    | SYNPILOI |
| 104            | 21                    | ZBBSR205 |
| 104            | 22                    | ZBBSR206 |
| 104            | 23                    | ZBBSR235 |
| 104            | 24                    | ZBBSR218 |
| 104            | 25                    | ZBBSR217 |
| 104            | 26                    | ZBBSR211 |
| 104            | 27                    | Pchroma  |
| 104            | 28                    | EUGROTUN |
| 104            | 29                    | ENTOGING |
| 104            | 30                    | DICDISCO |
| 104            | 31                    | ZTnucl   |
| 104            | 32                    | W10279   |
| 104            | 33                    | W10274   |
| 104            | 34                    | W10272   |
| 104            | 35                    | W10210   |
| 104            | 36                    | ZBBSR7   |
| 104            | 37                    | ZBBSR173 |
| 104            | 38                    | Siphycan |
| 104            | 39                    | ZBBSR2   |
| 104            | 40                    | ZCR4     |
| 104            | 41                    | ZCR16    |
| 104            | 42                    | ZCR6A    |
| 104            | 43                    | PHYPOLYC |
| 104            | 44                    | NAGRUBE  |
| 104            | 45                    | euk heli |
| 104            | 46                    | rad      |

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:     |
|----------------|-----------------------|-----------|
| 204            | 1                     | 1369mask  |
| 204            | 2                     | MNELEIDY  |
| 204            | 3                     | DIAGRAN   |
| 204            | 4                     | ATHBOMBA  |
| 204            | 5                     | BLAEMERS  |
| 204            | 6                     | CHLRINH   |
| 204            | 7                     | ORYSATIV  |
| 204            | 8                     | PORAERUG  |
| 204            | 9                     | ACACAST1  |
| 204            | 10                    | PHRBALAM  |
| 204            | 11                    | STYALSID  |
| 204            | 12                    | EMIHUXLE  |
| 204            | 13                    | OCHDANIC  |
| 204            | 14                    | CAFROENB  |
| 204            | 15                    | LABMINUT  |
| 204            | 16                    | OXYGRANTU |
| 204            | 17                    | BLEAMERI  |
| 204            | 18                    | HARVERMI  |
| 204            | 19                    | THEANNUL  |
| 204            | 20                    | SYMPTLOI  |
| 204            | 21                    | ZBBSR205  |
| 204            | 22                    | ZBBSR206  |
| 204            | 23                    | ZBBSR235  |
| 204            | 24                    | ZBBSR218  |
| 204            | 25                    | ZBBSR217  |
| 204            | 26                    | ZBBSR211  |
| 204            | 27                    | Pchroma   |
| 204            | 28                    | EUGROTUN  |
| 204            | 29                    | ENTOGING  |
| 204            | 30                    | DICDISCO  |
| 204            | 31                    | ZTnucl    |
| 204            | 32                    | W10279    |
| 204            | 33                    | W10274    |
| 204            | 34                    | W10272    |
| 204            | 35                    | W10210    |
| 204            | 36                    | ZBBSR7    |
| 204            | 37                    | ZBBSR173  |
| 204            | 38                    | Siphcyan  |
| 204            | 39                    | ZBBSR2    |
| 204            | 40                    | ZCR4      |
| 204            | 41                    | ZCR16     |
| 204            | 42                    | ZCR6A     |
| 204            | 43                    | PHYPOLYC  |
| 204            | 44                    | NAEGRUBE  |
| 204            | 45                    | euk heli  |
| 204            | 46                    | rad       |

"Sept4aln" on 5-SEP-96 15:33:41

| Position: | Sequence    | Data:  |
|-----------|-------------|--|
| 304       | 1 1369mask  | -----  |
| 304       | 2 MNELEIDY  | AAA-accAAU---gcguu-UAAC-----   |
| 304       | 3 DIAGRAND  | AAA-accAAc---AUU-----  |
| 304       | 4 ATHROMBA  | AAA-accAAc---gcgcUC-----   |
| 304       | 5 BLAEMERS  | AAA-accAAc---ccgcGCA-----  |
| 304       | 6 CHLREINH  | AAAG-gcAGC---cgggcUCU-----   |
| 304       | 7 ORYSATIV  | AAAG-gcAGCg---cgggcUCC-----  |
| 304       | 8 FORAERUG  | CGCA-accAGC---cggcUU--g-----   |
| 304       | 9 ACACAST1  | AAA-accAGCg---gcagggucaGC-----   |
| 304       | 10 PHRBALAM | UCAC-AgugcccccGAAcUGAGGCGUUCGACGUGGAGGAGACGCGUAGAAACAAcUGggguuuAAAAcCAAGGAGAAACAA----- |
| 304       | 11 STYALSID | CAAA-accAAc---ggcUU-----   |
| 304       | 12 EMIHUXLE | AGAA-ACcaa-A---CCGGU-----  |
| 304       | 13 OCHDANTC | -GAA-ACCaug---gggG-----  |
| 304       | 14 CAFROENB | -CAA-CCUUC-----  |
| 304       | 15 LABMINUT | -GAA-ACCaug---caggGUU-----   |
| 304       | 16 OXYGRANU | ACAA-ACCaaua---UUCCCG-----   |
| 304       | 17 BLEAMERI | AAA---CCCAacg---gggCGA-----  |
| 304       | 18 HARVERMI | UAAAaccGAC---accucucGC-----  |
| 304       | 19 THEANNUL | UAAA-accAA-a---cggcUU-----   |
| 304       | 20 SYMFILOI | CAGA-accAUG---caggCUC-----   |
| 304       | 21 ZBBSR205 | UACC-aucaug---CU-CUUC-----   |
| 304       | 22 ZBBSR206 | UACC-acCaug---CU-CUUC-----   |
| 304       | 23 ZBBSR235 | UACC-acCaug---CU-CUUC-----   |
| 304       | 24 ZBBSR218 | CCCC-acCaug---CC-UUU-----  |
| 304       | 25 ZBBSR217 | CCCC-acCaug---CC-UUU-----  |
| 304       | 26 ZBBSR211 | CCCC-acCaug---CC-CAU-----  |
| 304       | 27 Fchroma  | AAA-accAAcG---cgucucC-----   |
| 304       | 28 EUGROTUN | CAAA-accAAua---ccaccUC-----  |
| 304       | 29 ENTOTING | aUGUAGAAAG-----  |
| 304       | 30 DICDISCO | UCU---AccaUg---ccUU-----   |
| 304       | 31 ZTnucl   | gagU-aucaAAA---GUA-----  |
| 304       | 32 W10279   | gagU-aucaAAA---GUG-----  |
| 304       | 33 W10274   | gagU-aucaAAA---GUA-----  |
| 304       | 34 W10272   | gagU-aucaAAA---GUG-----  |
| 304       | 35 W10210   | gagU-aucaAAA---GUG-----  |
| 304       | 36 ZBBSR7   | GAAA-aucaACA-UGUACG-----   |
| 304       | 37 ZBBSR173 | auCaacUAAA---UGUG-----   |
| 304       | 38 Siphcyan | auUaaauUAAA---UUUA-----  |
| 304       | 39 ZBBSR2   | auCaacCAAAA---UGUU-----  |
| 304       | 40 ZCR4     | UuCaaACCAAAA---UGUU-----   |
| 304       | 41 ZCR16    | auUAGcCAGAG-UGAG-----  |
| 304       | 42 ZCR6A    | auCaacCAAAA---UGUG-----  |
| 304       | 43 PHYPOLYC | CAGG-UCGCAAA-UATUACUG-----   |
| 304       | 44 NAERGRUB | GCCUAgcuauug---UAaccuAGUUUuc-----  |
| 304       | 45 euk heli | 2  |
| 304       | 46 rad      | ----->   |

\*Sept4aln\* on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data: |
|----------------|-----------------------|-------|
| 404 1          | 1369mask              | ----- |
| 404 2          | MNELEIDY              | ----- |
| 404 3          | DIAGRAND              | ----- |
| 404 4          | ATHBOMBA              | ----- |
| 404 5          | BLAEMERS              | ----- |
| 404 6          | CHLREINH              | ----- |
| 404 7          | ORYSATIV              | ----- |
| 404 8          | PORAERUG              | ----- |
| 404 9          | ACACAST1              | ----- |
| 404 10         | PHRBALAM              | ----- |
| 404 11         | STYALSID              | ----- |
| 404 12         | EMIHUXLE              | ----- |
| 404 13         | OCHDANIC              | ----- |
| 404 14         | CAFRQENB              | ----- |
| 404 15         | LABMINUT              | ----- |
| 404 16         | OXYGRANU              | ----- |
| 404 17         | BLEAMERI              | ----- |
| 404 18         | HARVERMI              | ----- |
| 404 19         | THEANNUL              | ----- |
| 404 20         | SYMFILOI              | ----- |
| 404 21         | ZBBSR205              | ----- |
| 404 22         | ZBBSR206              | ----- |
| 404 23         | ZBBSR235              | ----- |
| 404 24         | ZBBSR218              | ----- |
| 404 25         | ZBBSR217              | ----- |
| 404 26         | ZBBSR211              | ----- |
| 404 27         | Pchroma               | ----- |
| 404 28         | EUGROTUN              | ----- |
| 404 29         | ENTOGING              | ----- |
| 404 30         | DICDISCO              | ----- |
| 404 31         | Ztnucl                | ----- |
| 404 32         | W10279                | ----- |
| 404 33         | W10274                | ----- |
| 404 34         | W10272                | ----- |
| 404 35         | W10210                | ----- |
| 404 36         | ZBBSR7                | ----- |
| 404 37         | ZBBSR173              | ----- |
| 404 38         | Siphcyan              | ----- |
| 404 39         | ZBBSR2                | ----- |
| 404 40         | ZCR4                  | ----- |
| 404 41         | ZCR16                 | ----- |
| 404 42         | ZCR6A                 | ----- |
| 404 43         | PHYPOLYC              | ----- |
| 404 44         | NAEGRUBE              | ----- |
| 404 45         | euk heli              | ----- |
| 404 46         | rad                   | ----- |



"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:  |
|----------------|-----------------------|--|
| 504            | 1 1369mask            | -----  |
| 504            | 2 MNELEIDY            | -----gacgc-----UUUCgguGauUucAua-auaaCU   |
| 504            | 3 DIAGRAND            | -----UUUCuugguGAuUucAua-auaaCU   |
| 504            | 4 ATHBOMBA            | -----gacgc-----UCCYugguGAuUucAua-auaaCU  |
| 504            | 5 BLAEMERS            | -----Acggg-UUUUcugguGAuUucAua-auaaCU   |
| 504            | 6 CHLREINH            | -----gcccg-----ACCUcgguGAuUucAua-auaaCU  |
| 504            | 7 ORYSATIV            | -----gcccg-----CUUAUcguGAuUucAua-auaaCU  |
| 504            | 8 PORAEFRUG           | -----ccc--g-----UUUu-gguGAGuUa-auaaCU  |
| 504            | 9 ACACASTI            | -----AA-ugggcccu-gccAA-----ACACUCcugguGAuUucAua-auaaCU                           |
| 504            | 10 PHRBALAM           | -----AACCGAAAGCAACUCUAUCAGUUGGUGGAGUCCGUUAUCCUGCGGCCAGCGUUGGAGUCCGAAcGAggGcauuCA |
| 504            | 11 STYALSID           | -----gccgu-----UUUuugguGAUUAUg-guaaCU  |
| 504            | 12 EMIHUXLE           | -----cUCC--GG-----nugCGUGUGAGuUucAua-auaaCU                                      |
| 504            | 13 OCHDANIC           | -----CAAcccu-----UUAUUGGUGAGuUucAua-auaaCU                                       |
| 504            | 14 CAFROENB           | -----AACACAGGUGAGuUucAua-auaaCU  |
| 504            | 15 LABMINUT           | -----UcccuGg-----UguuUGGUGAGuUucAua-auaaCU                                       |
| 504            | 16 OXYGRANU           | -----UGUC-----UuuUGGAGGAGuUucAua-auaaCU  |
| 504            | 17 BLEAMERI           | -----C-----ccugUGGAGGAGuUucAua-auaaCU  |
| 504            | 18 HARVERMI           | -----GAggggaggu-----AGCACAgguGAUucAua-auaaCU                                     |
| 504            | 19 THEANNUL           | -----gcgguGU-----CCggguGAUucAua-auaaCU   |
| 504            | 20 SYMPTLOI           | -----UGccu-----gUUUGgguGAUucAua-auaaCU   |
| 504            | 21 ZBBSR205           | -----GGAGCUUcGAGuUucAua-auaaCU   |
| 504            | 22 ZBBSR206           | -----GGAGCUUcGAGuUucAua-auaaCU   |
| 504            | 23 ZBBSR235           | -----GGAGCUUcGAGuUucAua-auaaCU   |
| 504            | 24 ZBBSR218           | -----AUGGCUUcGAGuUucAua-auaaCU   |
| 504            | 25 ZBBSR217           | -----AUGGCUUcGAGuUucAua-auaaCU   |
| 504            | 26 ZBBSR211           | -----UGGCUUcGAGuUucAua-auaaCU  |
| 504            | 27 Pchroma            | -----UgggggGUUUGGUGGAGuUucAua-auaaCU   |
| 504            | 28 EUGROTUN           | -----ggugg-----CUuuUGGUGAGuUucAua-auaaCU   |
| 504            | 29 ENTOTING           | -----AAGUUUAUUAAGAAAG-AACAA  |
| 504            | 30 DICDISCO           | -----CG-----ggUUuugGUGuUucAua-auaaCU   |
| 504            | 31 ZTnucl             | -----CGACUUAUC-ugaAUUcuaAUa-uuuAC-   |
| 504            | 32 W10279             | -----CGACUUAUC-ugaAUUcuaAUa-uuuAC-   |
| 504            | 33 W10274             | -----CGACUUAUC-ugaAUUcuaAUa-uuuAC-   |
| 504            | 34 W10272             | -----CGACUUAUC-ugaAUUcuaAUa-uuuAC-   |
| 504            | 35 W10210             | -----CGACUUAUC-ugaAUUcuaAUa-uuuAC-   |
| 504            | 36 ZBBSR7             | -----GCAUAUUGGUGA-ugaauUcuaAUa-UcaAUU  |
| 504            | 37 ZBBSR173           | -----CGUACAUAUUGA-AGguuuuaAUa-UUUAUUA  |
| 504            | 38 Siphcyan           | -----UAUAUUAUUGA-GGAuuUuaAUa-UuaCUU  |
| 504            | 39 ZBBSR2             | -----UCAGCAUAUUGA-UgAUuCauguaa-UguUU-  |
| 504            | 40 ZCR4               | -----UUAUCAUAUUGA-UgAUuCauguaa-UguUU-  |
| 504            | 41 ZCR16              | -----UUAUAUAUUGA-UgaauCaUuaAUa-UU-CU-  |
| 504            | 42 ZCR6A              | -----UGUACAUA-UG-----GAAGguuuuaAUa-UUGUU-  |
| 504            | 43 PHYPOLYC           | -----GGAGUGGcc-----acagcaucUGACCACUAU-CCAAAC                                     |
| 504            | 44 NAEGRUBE           | -----ggGUGUGGCAACAUAUUCGGG-----GAUUAAGAAUCCGCCUAGCAGGUG                          |
| 504            | 45 euk heli           | 12   |
| 504            | 46 rad                | rad  |





[illegible]

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>Identity: | Data:     |
|----------------|-----------------------|-----------|
| 906            | 1                     | 1369mask  |
| 906            | 2                     | MNELEIDY  |
| 906            | 3                     | DIAGRAN   |
| 906            | 4                     | ATHBOMBA  |
| 906            | 5                     | BLAEMERS  |
| 906            | 6                     | CHLREINH  |
| 906            | 7                     | ORYSATIV  |
| 906            | 8                     | PORABRUG  |
| 906            | 9                     | ACACAST1  |
| 906            | 10                    | PHRBALAM  |
| 906            | 11                    | STYALSID  |
| 906            | 12                    | EMIHUXLE  |
| 906            | 13                    | OCHDANIC  |
| 906            | 14                    | CAPROENB  |
| 906            | 15                    | LABMINUT  |
| 906            | 16                    | OXYGRANU  |
| 906            | 17                    | BLAEMERI  |
| 906            | 18                    | HARVERMI  |
| 906            | 19                    | THEANNUL  |
| 906            | 20                    | SYMPTILO1 |
| 906            | 21                    | ZBBSR205  |
| 906            | 22                    | ZBBSR206  |
| 906            | 23                    | ZBBSR235  |
| 906            | 24                    | ZBBSR218  |
| 906            | 25                    | ZBBSR217  |
| 906            | 26                    | ZBBSR211  |
| 906            | 27                    | Pchroma   |
| 906            | 28                    | EUGROTUN  |
| 906            | 29                    | ENTOTING  |
| 906            | 30                    | DICDISCO  |
| 906            | 31                    | Ztnucl    |
| 906            | 32                    | W10279    |
| 906            | 33                    | W10274    |
| 906            | 34                    | W10272    |
| 906            | 35                    | W10210    |
| 906            | 36                    | ZBBSR7    |
| 906            | 37                    | ZBBSR173  |
| 906            | 38                    | Siphcyan  |
| 906            | 39                    | ZBBSR2    |
| 906            | 40                    | ZCR4      |
| 906            | 41                    | ZCR16     |
| 906            | 42                    | ZCR6A     |
| 906            | 43                    | PHYPOLYC  |
| 906            | 44                    | NAEGRUBE  |
| 906            | 45                    | euk hell  |
| 906            | 46                    | rad       |

[illegible]

"Sept4aln" on 5-SEP-96 15:33:41

| Position | Sequence Identity | Data                                       |
|----------|-------------------|--|
| 1107 1   | 1369mask          | -----                                      |
| 1107 2   | MNELEIDY          | -----CCGGCC-G-A-UUGGUCCG-----              |
| 1107 3   | DIAGRAN           | -----ACUGAGG-G-GUGUCCGCC-----              |
| 1107 4   | ATHROMBA          | -----CUGGUU-G-GCGGUCCGCU-----              |
| 1107 5   | BLAEMERS          | -----CUGUCUUGU-C-A-CCUGGCCACU-----         |
| 1107 6   | CHLREINH          | -----GGGUG-G-U-CCGGUCCGCC-----             |
| 1107 7   | ORYSATIV          | -----GGGCGG-G-CCGGUCCGCC-----              |
| 1107 8   | PORAERUG          | -----GGU-GAS-A-UYGUGU-----GG               |
| 1107 9   | ACACAST1          | -----CGCGCAU-U-CAAGCGCC-----GUGUGUCCG      |
| 1107 10  | PHRBALAM          | -----CUAUGCCUUAUCCCGCCA-----U              |
| 1107 11  | STYALSID          | -----CCGG-GAC-A-CGGUCC-----                |
| 1107 12  | EMIHUXLE          | -----CGGG-CCG-A-CCGGUCUGC-----             |
| 1107 13  | OCHDANIC          | -----UUCG-CAU-G-UCGGUCCGCC-----            |
| 1107 14  | CAFRONB           | -----CUG-CCG-AGNC-GGGGNCU-----             |
| 1107 15  | LABMINUT          | -----AGGA-GUG-A-CCGUCCGAAU-----            |
| 1107 16  | OXYGRANU          | -----AGGUGCC-A-AUGUCCGUCGUUG-----          |
| 1107 17  | BLEAMERI          | -----GGGCGGUC-G-CUGGGCCCGGUCU-----         |
| 1107 18  | HARVERMI          | -----AGGUCUUA-GU--GGUCCG-----              |
| 1107 19  | THEANNUL          | -----                                      |
| 1107 20  | SYMPILOI          | -----AGGAUGA-----CCGGUCCGCCUUCU-----GG     |
| 1107 21  | ZBBSR205          | -----AGGUCAG--UU-UAGUCUUCAU-----           |
| 1107 22  | ZBBSR206          | -----AGGUCAG--UU-AAGUCU-CUUCU-----         |
| 1107 23  | ZBBSR235          | -----AGGUCAG--UU-UAGUCUUCAU-----           |
| 1107 24  | ZBBSR218          | -----AUUAGG-----U-----CCACCUACUC-----      |
| 1107 25  | ZBBSR217          | -----AUUAGG-----U-----CCACCUACUC-----      |
| 1107 26  | ZBBSR211          | -----AUUG-----CAGA--U--GCACACUUCU-----     |
| 1107 27  | Pchroma           | -----UUGA--CCGGG-CAC-----                  |
| 1107 28  | EUGROTUN          | -----GAACCAAUUGG-CCCGUCC-C-U-UU-----       |
| 1107 29  | ENTOGING          | -----                                      |
| 1107 30  | DICDISCO          | -----UUUA-----CCGGG-----UUUAUGU--CAUU----- |
| 1107 31  | ZTnucl            | -----                                      |
| 1107 32  | W10279            | -----                                      |
| 1107 33  | W10274            | -----                                      |
| 1107 34  | W10272            | -----                                      |
| 1107 35  | W10210            | -----                                      |
| 1107 36  | ZBBSR7            | -----                                      |
| 1107 37  | ZBBSR173          | -----                                      |
| 1107 38  | Siphcyan          | -----                                      |
| 1107 39  | ZBBSR2            | -----                                      |
| 1107 40  | ZCR4              | -----                                      |
| 1107 41  | ZCR16             | -----                                      |
| 1107 42  | ZCR6A             | -----                                      |
| 1107 43  | PHYPOLYC          | -----                                      |
| 1107 44  | NAEGRUBE          | -----                                      |
| 1107 45  | euk heli          | -----                                      |
| 1107 46  | rad               | -----                                      |

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:   |
|----------------|-----------------------|---|
| 1208 1         | 1369mask              | -----UCC-UCUG-----  |
| 1208 2         | MNELEIDY              | -----GAUCGUG-UACUGAUC-----  |
| 1208 3         | DIAGRAND              | -----UCA-----   |
| 1208 4         | ATHOMBBA              | -----U--AA-----   |
| 1208 5         | BLAEMERS              | -----GAGCUUURCGCCAGUG-----  |
| 1208 6         | CHLREINH              | -----UC-----  |
| 1208 7         | ORYSATIV              | -----UCA-----   |
| 1208 8         | PORAERUG              | GGCAACU-----  |
| 1208 9         | ACACAST1              | GUCAAAACCGGACUG---CG-UTG---CGUUGCGGC---UCGGUCCGUGGACCCUGGUCUUAU-UCGCGGAGCCGUA |
| 1208 10        | PHRBALAM              | -----GCUUUAUUGAGUCUUGGAUAC-----   |
| 1208 11        | STVALSID              | GGCAAGU-----  |
| 1208 12        | EMIHUXLE              | -----CGAU-----  |
| 1208 13        | OCHDANIC              | -----CAAG-----  |
| 1208 14        | CAFROENB              | -----   |
| 1208 15        | LAMINUT               | -----GAUUGUUGGUAUUGUUGUU-----   |
| 1208 16        | OXYGRANU              | -----CGUGUGCAGC-----  |
| 1208 17        | BLEAMERI              | -----GGU-----   |
| 1208 18        | HARVERMI              | -----   |
| 1208 19        | THEANNUL              | -----   |
| 1208 20        | SYMPILO1              | GUGUGAUCUGGU-----   |
| 1208 21        | ZBBSR205              | UGUAAGAUCUA-----  |
| 1208 22        | ZBBSR206              | AUUGAGUUCU-----   |
| 1208 23        | ZBBSR235              | UGUAAGAUCUA-----  |
| 1208 24        | ZBBSR218              | UGUUGUAGCGU-----  |
| 1208 25        | ZBBSR217              | UGUUGUAGCGU-----  |
| 1208 26        | ZBBSR211              | CGAGGCAGC-----  |
| 1208 27        | Pchroma               | GUCGCGUCCU-----   |
| 1208 28        | EUGROTUN              | -----   |
| 1208 29        | ENTOGING              | -----GU--GGUUA-----   |
| 1208 30        | DICDISCO              | -----U--ACCACUUC-----   |
| 1208 31        | ZTnucl                | -----RUUUUAUUUAUAUAG-UUAU-----  |
| 1208 32        | W10Z79                | -----AUUUUAUUUAUAUAG-UUAU-----  |
| 1208 33        | W10Z74                | -----AUUUUAUUUAUAUAG-UUAU-----  |
| 1208 34        | W10Z72                | -----AUUUUAUUUAUAUAG-UUAU-----  |
| 1208 35        | W10Z10                | -----AUUUUAUUUAUAUAG-UUAU-----  |
| 1208 36        | ZBBSR7                | -----GGAUCAAUUUUGUGUGG-UCUC-----  |
| 1208 37        | ZBBSR173              | -----UUAUAUUUUUAUGUGA-UCAU-----   |
| 1208 38        | Siphcyan              | -----UAUUCAAUUUUGUGUGG-UCCU-----  |
| 1208 39        | ZBBSR2                | -----GAAUCAAUUUUAUAGU-UUAU-----   |
| 1208 40        | ZCR4                  | -----AAUCAAUUUUUAUGUGA-UCCU-----  |
| 1208 41        | ZCR16                 | -----GAAUCAAUUUUUAUGUGA-UCCU-----   |
| 1208 42        | ZCR6A                 | -----UUUUUAUUUUUAUAGU-UCAU-----   |
| 1208 43        | PHYPOLYC              | -----UUGGACACUGGUGCAG-CUGC-----   |
| 1208 44        | NAEGRUBE              | -----UUAUUUUUACAGAGCGGUGGCGAGUUUAUC-----                                      |
| 1208 45        | euk heli              | -----ACUCUUG-----   |
| 1208 46        | rad                   | -----   |



"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:   |
|----------------|-----------------------|---|
| 1311           | 1 1369mask            | -----   |
| 1311           | 2 MNELEIDY            | -----GGUCUGUU-CUU-----CUUC  |
| 1311           | 3 DIAGRAND            | -----UCCUCUGUGACAU-----CCUC   |
| 1311           | 4 ATHBOMBA            | -----UGACUGG-GUCUU-----ACCU   |
| 1311           | 5 BLAEMERS            | -----GGGCCC--UTUAUUU-----CUGC   |
| 1311           | 6 CHUREINH            | -----UGUCC--ACCUUC-----CUGC   |
| 1311           | 7 ORYSATIV            | -----CUGCUG--ACCCUU-----CUGC  |
| 1311           | 8 PORAEUG             | -----CCGCC--GCCUU-----CCUC  |
| 1311           | 9 ACACAST1            | -----CU--CCUUCUG--GAUCCCGUUCU--CCUAUUGAGUAGUGGGGACGUCU-----CAGG                         |
| 1311           | 10 PHBALAM            | AGGAUUGGGGGAUAAGGACUAAGGUUACCUUGAACAAUAUAGUGAUCAGGCGUUUUUAAGCCUUGAUAUAGCAUAGGAGGACAAACA |
| 1311           | 11 STVALSID           | -----GGG--CCUUU-----UUCG  |
| 1311           | 12 EMTUXLE            | -----GGCGGUCUU-----CCACC  |
| 1311           | 13 OCHDANTIC          | -----GUUC--GGAUACU-----CCUCG  |
| 1311           | 14 CAFROENB           | -----CAA--GUUCAU-----C----  |
| 1311           | 15 LABMINUT           | -----CUCC--AGCCAU-----CCUU--  |
| 1311           | 16 OXYGRANU           | -----GGCGGCCUU--CCAU-----CCUUC  |
| 1311           | 17 BLEMERI            | -----GACCUUCUCUGAU-----CCAUC  |
| 1311           | 18 HARVERMI           | -----CCUUCGGGAGCGGUCACUGGCC--UCCUA-----UGUUC  |
| 1311           | 19 THEANNUL           | -----UGCAUUGUUGUCCU-----CUGGG   |
| 1311           | 20 SYMPIOLOI          | -----CAGCCUUGA--CAUCU-----CUGG  |
| 1311           | 21 ZBBSR205           | -----UAUGGGCCUG-----CUUCU   |
| 1311           | 22 ZBBSR206           | -----UAUGGGCCUG-----CUUCU   |
| 1311           | 23 ZBBSR235           | -----UAUGGGCCUG-----CUUCU   |
| 1311           | 24 ZBBSR218           | -----GGCUCUUUA--CUUCU-----  |
| 1311           | 25 ZBBSR217           | -----GGCUCUUUA--CUUCU-----  |
| 1311           | 26 ZBBSR211           | -----ACUCUGAU--CUUCU-----   |
| 1311           | 27 Pchroma            | -----UCCUGCGUUUGCCAUCC  |
| 1311           | 28 EUGROTUN           | -----GUGCCGCUUGAGUUUGUCAU-----CCUUC   |
| 1311           | 29 ENTOTING           | -----UUGAAAAG--CUUUU  |
| 1311           | 30 DICDISCO           | -----AUGACACCGUUCUCUUU-----CUUAA  |
| 1311           | 31 ZTrucl             | -----AUGUA--CUAUAUUGACAUGCCU-----AAAUC  |
| 1311           | 32 W10Z79             | -----AUGUA--CUAUAUUGACAUGCCU-----AAAUC  |
| 1311           | 33 W10Z74             | -----AUGUA--CUAUAUUGACAUGCCU-----AAAUC  |
| 1311           | 34 W10Z72             | -----AUGUA--CUAUAUUGACAUGCCU-----AAAUC  |
| 1311           | 35 W10Z10             | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 36 ZBBSR7             | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 37 ZBBSR173           | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 38 Siphycan           | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 39 ZBBSR2             | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 40 ZCR4               | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 41 ZCR16              | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 42 ZCR6A              | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 43 PHYPOLYC           | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 44 NAGRUBE            | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 45 euk heli           | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |
| 1311           | 46 rad                | -----AAUGA--CG--GCAAGAAAUU-----GAU-----UUUUC  |

"Sept4a1n" on 5-SEP-96 15:33:41

[illegible]

[illegible]



"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>Identity: | Data:    |
|----------------|-----------------------|----------|
| 1712           | 1                     | 1369mask |
| 1712           | 2                     | MNLEIDY  |
| 1712           | 3                     | DIAGRAN  |
| 1712           | 4                     | ATHBOMBA |
| 1712           | 5                     | BLAEMERS |
| 1712           | 6                     | CHLREINH |
| 1712           | 7                     | ORYSATIV |
| 1712           | 8                     | FORAERUG |
| 1712           | 9                     | ACACAST1 |
| 1712           | 10                    | PHRBALAM |
| 1712           | 11                    | STYALSID |
| 1712           | 12                    | EMTHUXLE |
| 1712           | 13                    | OCHDANIC |
| 1712           | 14                    | CAPROENB |
| 1712           | 15                    | LAMINUT  |
| 1712           | 16                    | OXYGRANU |
| 1712           | 17                    | BLEAMERI |
| 1712           | 18                    | HARVERMI |
| 1712           | 19                    | THEANNUL |
| 1712           | 20                    | SYMPILO1 |
| 1712           | 21                    | ZBBSR205 |
| 1712           | 22                    | ZBBSR206 |
| 1712           | 23                    | ZBBSR235 |
| 1712           | 24                    | ZBBSR218 |
| 1712           | 25                    | ZBBSR217 |
| 1712           | 26                    | ZBBSR211 |
| 1712           | 27                    | Pchroma  |
| 1712           | 28                    | EUGROTUN |
| 1712           | 29                    | ENTOGING |
| 1712           | 30                    | DICDISCO |
| 1712           | 31                    | ZTnucl   |
| 1712           | 32                    | W10Z79   |
| 1712           | 33                    | W10Z74   |
| 1712           | 34                    | W10Z72   |
| 1712           | 35                    | W10Z10   |
| 1712           | 36                    | ZBBSR7   |
| 1712           | 37                    | ZBBSR173 |
| 1712           | 38                    | Siphcyan |
| 1712           | 39                    | ZBBSR2   |
| 1712           | 40                    | ZCR4     |
| 1712           | 41                    | ZCR16    |
| 1712           | 42                    | ZCR6A    |
| 1712           | 43                    | PHYPOLYC |
| 1712           | 44                    | NASGRUBE |
| 1712           | 45                    | euk heli |
| 1712           | 46                    | rad      |



\*Sept4aln\* on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:    |
|----------------|-----------------------|----------|
| 1912           | 1                     | 1369mask |
| 1912           | 2                     | MNELEIDY |
| 1912           | 3                     | DIAGRAN  |
| 1912           | 4                     | ATHBOMBA |
| 1912           | 5                     | BLAEMERS |
| 1912           | 6                     | CHLREINH |
| 1912           | 7                     | ORYSATIV |
| 1912           | 8                     | PORAERUG |
| 1912           | 9                     | ACACAST1 |
| 1912           | 10                    | PHRBALAM |
| 1912           | 11                    | STYALSID |
| 1912           | 12                    | EMTHUXLE |
| 1912           | 13                    | OCHDANIC |
| 1912           | 14                    | CAPROENB |
| 1912           | 15                    | LABMINUT |
| 1912           | 16                    | OXYGRANU |
| 1912           | 17                    | BLEMERI  |
| 1912           | 18                    | HARVERMI |
| 1912           | 19                    | THEANNUL |
| 1912           | 20                    | SYNPILO1 |
| 1912           | 21                    | ZBBSR205 |
| 1912           | 22                    | ZBBSR206 |
| 1912           | 23                    | ZBBSR235 |
| 1912           | 24                    | ZBBSR218 |
| 1912           | 25                    | ZBBSR217 |
| 1912           | 26                    | ZBBSR211 |
| 1912           | 27                    | Pchroma  |
| 1912           | 28                    | EUGROTUN |
| 1912           | 29                    | ENTOGING |
| 1912           | 30                    | DICDISCO |
| 1912           | 31                    | ZTnucl   |
| 1912           | 32                    | W10279   |
| 1912           | 33                    | W10274   |
| 1912           | 34                    | W10272   |
| 1912           | 35                    | W10210   |
| 1912           | 36                    | ZBBSR7   |
| 1912           | 37                    | ZBBSR173 |
| 1912           | 38                    | Siphcyan |
| 1912           | 39                    | ZBBSR2   |
| 1912           | 40                    | ZCR4     |
| 1912           | 41                    | ZCR16    |
| 1912           | 42                    | ZCR6A    |
| 1912           | 43                    | PHYPOLYC |
| 1912           | 44                    | NABGRUBE |
| 1912           | 45                    | euk heli |
| 1912           | 46                    | rad      |

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:    |
|----------------|-----------------------|----------|
| 2012           | 1                     | 1369mask |
| 2012           | 2                     | MNELEIDY |
| 2012           | 3                     | DIAGRAN  |
| 2012           | 4                     | ATHBOMBA |
| 2012           | 5                     | BLAEMERS |
| 2012           | 6                     | CHUREINH |
| 2012           | 7                     | ORYSATIV |
| 2012           | 8                     | PORAERUG |
| 2012           | 9                     | ACACAST1 |
| 2012           | 10                    | PHRBALAM |
| 2012           | 11                    | STYALSID |
| 2012           | 12                    | EMTHUXLE |
| 2012           | 13                    | OCHDANIC |
| 2012           | 14                    | CAFROENB |
| 2012           | 15                    | LABMINUT |
| 2012           | 16                    | OXYGRANU |
| 2012           | 17                    | BLEAMERI |
| 2012           | 18                    | HARVERMI |
| 2012           | 19                    | THEANNUL |
| 2012           | 20                    | SYNPIL01 |
| 2012           | 21                    | ZBBSR205 |
| 2012           | 22                    | ZBBSR206 |
| 2012           | 23                    | ZBBSR235 |
| 2012           | 24                    | ZBBSR218 |
| 2012           | 25                    | ZBBSR217 |
| 2012           | 26                    | ZBBSR211 |
| 2012           | 27                    | Pchroma  |
| 2012           | 28                    | EUGROTUN |
| 2012           | 29                    | ENTOGING |
| 2012           | 30                    | DICDISCO |
| 2012           | 31                    | ZTnucl   |
| 2012           | 32                    | W10279   |
| 2012           | 33                    | W10274   |
| 2012           | 34                    | W10272   |
| 2012           | 35                    | W10210   |
| 2012           | 36                    | ZBBSR7   |
| 2012           | 37                    | ZBBSR173 |
| 2012           | 38                    | Siphcyan |
| 2012           | 39                    | ZBBSR2   |
| 2012           | 40                    | ZCR4     |
| 2012           | 41                    | ZCR16    |
| 2012           | 42                    | ZCR6A    |
| 2012           | 43                    | PHYPOLYC |
| 2012           | 44                    | NAGRUBE  |
| 2012           | 45                    | euk hell |
| 2012           | 46                    | rad      |





"Sept4aln" on 5-SEP-96 15:33:41

[illegible]

"Sept4a1n" on 5-SEP-96 15:33:41

[illegible]

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:   |
|----------------|-----------------------|---|
| 2413           | 1 1369mask            | 111-1111-----   |
| 2413           | 2 MNELEIDY            | uaa-AUagugA--cacgguuC-----  |
| 2413           | 3 DIAGRAND            | uaa-AUaguuA--cacGauuu-----  |
| 2413           | 4 ATBOMBA             | uaa-AUagacA--ggcgggcU-----  |
| 2413           | 5 BLAEMERS            | uaa-UUaguuG--ggcuugcaUU-----  |
| 2413           | 6 CHLREINH            | uaa-AUagucA--gcaucgc-----   |
| 2413           | 7 ORYSATIV            | uaa-CUagcua--ugcggagC-----  |
| 2413           | 8 FORAERUG            | uaa-CUagugG--auugaa-----ucu-----  |
| 2413           | 9 ACACAST1            | uaa-AUAugcC--gcguaacccGUCCAUAAACCAUGCGUGGucacgcgcUgcgggugguugucgu-----UCGC-----                   |
| 2413           | 10 PHRBALAM           | uaa-AUAUGUUGGC--AGAGGCCUUUGUUGauuAAGuuuuuuuuuUUGGGGCAACUCGGUuagaggggaugagucUUUAUCAAACUCUAGUuucaaa |
| 2413           | 11 STYALSID           | uaa-AUagcgA--cgcgaa-----  |
| 2413           | 12 EMTHUXLE           | uaa-CUagucG--UcuGaaugC-----   |
| 2413           | 13 OCHDANIC           | uaa-CUagucG--UcuGaaugC-----   |
| 2413           | 14 CAFROENB           | uagUCUagauG--uuugcuC-----   |
| 2413           | 15 LAEMINUT           | uaa-AUagU--G-ugcauuuuc-----   |
| 2413           | 16 OXYGRANU           | uaa-CUagucGAAC--c-aaUCUC-----   |
| 2413           | 17 BLEAMERI           | uaa-CUagucG-UcucuugCC-----  |
| 2413           | 18 HARVERMI           | uaa-AUaguc-A-cgcgaacCggucgcGCA-----   |
| 2413           | 19 THEANNUL           | uaa-AUag--ggucgggAauaAGUU-----  |
| 2413           | 20 SYMPIOLO           | uaa-AUag--uuAcauguuaaccUC-----  |
| 2413           | 21 ZBBSR205           | uaa-AUagU--gucauuuuuuc-----   |
| 2413           | 22 ZBBSR206           | uaa-AUagU--gucauuuuuuc-----   |
| 2413           | 23 ZBBSR235           | uaa-AUagU--gucauuuuuuc-----   |
| 2413           | 24 ZBBSR218           | uaa-AUag--uuagauu--cug-----   |
| 2413           | 25 ZBBSR217           | uaa-AUag--uuagauu--cug-----   |
| 2413           | 26 ZBBSR211           | uaa-AUaguc--acaUcu-cu-----  |
| 2413           | 27 Fchroma            | uaa-AUagggggccGaaucU-----   |
| 2413           | 28 EUGROTUN           | uaa-AUaguu--cuUgucac-----   |
| 2413           | 29 ENTOTING           | uaa-UUagUUGcauuuGaaugGAAAUGCA-----  |
| 2413           | 30 DICDISCO           | uaa-CUaguaG-UAUU-UAUUAGUCGAUAUAGACGAUAGUUAUUGGGGUUGGAAUGAUUUGGUAUCUCCU-----                       |
| 2413           | 31 ZTrucl             | uaa-aUaguaa--gyacugCA-----  |
| 2413           | 32 W10279             | uaa-aUaguaa--ggcuguaU-----  |
| 2413           | 33 W10274             | uaa-aUaguaa--guacugu-----   |
| 2413           | 34 W10272             | uaa-aUaguaa--ggcuaUGU-----  |
| 2413           | 35 W10210             | uaa-aUaguaa--gcGcuguGU-----   |
| 2413           | 36 ZBBSR7             | uaa-AUagcaG--Uaaauuuu-----  |
| 2413           | 37 ZBBSR173           | UUA-UUCgcaU--C-aaauU-----   |
| 2413           | 38 Siphcyan           | Uaa-UUCgcuU--aaauaUU-----   |
| 2413           | 39 ZBBSR2             | uaa-AUagucC--auuuuuU-----   |
| 2413           | 40 ZCR4               | uaa-AUggcaC--aUuu-----  |
| 2413           | 41 ZCR16              | uaa-AUaguu--aaucAC-----   |
| 2413           | 42 ZCR6A              | Uaa-UUCguauU-----uaauaU-----  |
| 2413           | 43 PHYPOLYC           | uaa-UAGGGG--GGC-AGCCAGCCGUCGCCAGACAGGUUAGCUC-----   |
| 2413           | 44 NAERGRUBE          | uaa-CUAGCCGUAAGC-----CUUUCUUGGGGAAGGUUAGUUUUGCGAACAGGUUUC-----                                    |
| 2413           | 45 euk heli           | 5-0   |
| 2413           | 46 rad                | ----> ----->>   |

| Posi-<br>tion: | Sequence<br>identity: | Data:     |
|----------------|-----------------------|-----------|
| 2513           | 1                     | 1369mask  |
| 2513           | 2                     | MNELEIDY  |
| 2513           | 3                     | DIAGRAND  |
| 2513           | 4                     | ATHBOMBA  |
| 2513           | 5                     | BLAEMERS  |
| 2513           | 6                     | CHLRINH   |
| 2513           | 7                     | ORYSATIV  |
| 2513           | 8                     | FORAERUG  |
| 2513           | 9                     | ACACAST1  |
| 2513           | 10                    | PHRBALAN  |
| 2513           | 11                    | STYVALSID |
| 2513           | 12                    | EMTHUXLE  |
| 2513           | 13                    | OCHDANIC  |
| 2513           | 14                    | CAFROENB  |
| 2513           | 15                    | LAMBTINUT |
| 2513           | 16                    | OXYGRANU  |
| 2513           | 17                    | BLEAMERI  |
| 2513           | 18                    | HARVERMI  |
| 2513           | 19                    | THEANNUL  |
| 2513           | 20                    | SYMPTILO1 |
| 2513           | 21                    | ZBBSR205  |
| 2513           | 22                    | ZBBSR206  |
| 2513           | 23                    | ZBBSR235  |
| 2513           | 24                    | ZBBSR218  |
| 2513           | 25                    | ZBBSR217  |
| 2513           | 26                    | ZBBSR211  |
| 2513           | 27                    | Pchroma   |
| 2513           | 28                    | EUGROTUN  |
| 2513           | 29                    | ENTONGING |
| 2513           | 30                    | DICDLSGO  |
| 2513           | 31                    | Ztnucl    |
| 2513           | 32                    | W10279    |
| 2513           | 33                    | W10274    |
| 2513           | 34                    | W10272    |
| 2513           | 35                    | W10210    |
| 2513           | 36                    | ZBBSR7    |
| 2513           | 37                    | ZBBSR173  |
| 2513           | 38                    | Siphcyan  |
| 2513           | 39                    | ZBBSR2    |
| 2513           | 40                    | ZCR4      |
| 2513           | 41                    | ZCR16     |
| 2513           | 42                    | ZCR6A     |
| 2513           | 43                    | PHYPOLYC  |
| 2513           | 44                    | NAEGRUBE  |
| 2513           | 45                    | euk heli  |
| 2513           | 46                    | rad       |

"Sept4ain" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:   |
|----------------|-----------------------|---|
| 2613           | 1 1369mask            | 111-----111111111   |
| 2613           | 2 MNELEIDY            | uau-cg-gu-----uUcAaacgauggaAGUu   |
| 2613           | 3 DIAGRAN             | uau-cg-gu-----GUUuacccauggaAGUu   |
| 2613           | 4 ATHBOMBA            | ugu-ca-gc-----GUCUagcugagcgaAGUu  |
| 2613           | 5 BLAEMERS            | ugc-ug-gc-----GUUuagccagaggAAGUu  |
| 2613           | 6 CHLREINH            | uau-ug-gc-----GUUuagcccauggAAGUa  |
| 2613           | 7 ORYSATIV            | uAu-gg-cc-----GUUuagggccaCggaAGUu   |
| 2613           | 8 FORAERUG            | ACc-cu-gg-----G-CAaccaggaggAGGUu  |
| 2613           | 9 ACACAST1            | ugc-ug-gc-----GCCUagccagcggAAGUu  |
| 2613           | 10 PHRBALAM           | uau-uu-gcUC-----CCAgcaaauggAAGUu  |
| 2613           | 11 STYALSID           | uau-cgUGAU-----UACCAacggauggAAGUu   |
| 2613           | 12 EMIHUXLE           | Aac-uu-gu-----CUUCAacaaguGgAAGUu  |
| 2613           | 13 OCHDANIC           | uuu-ug-gu-----GACUaaccacaaggAAGUu   |
| 2613           | 14 CAFROENB           | uau-gg-uc-----GUUuagggccauggAAGUu   |
| 2613           | 15 LABMINUT           | Auu-uc-gg-----GCCAAacacaaggAAGUu  |
| 2613           | 16 OXYGRANU           | uuu-gu-gg-----GCCUacuccaaggAAGUu  |
| 2613           | 17 BLEAMERI           | uuu-g--gg-----CCAgcgauggAAGUu   |
| 2613           | 18 HARVERMI           | uau-ugcCACCG-----AUAauucgaaggAAGUu  |
| 2613           | 19 THEANNUL           | uuu-gc-gguU-----GUCUacgcaaggAAGUu   |
| 2613           | 20 SYMFILO1           | uuu-gu-gu-----AACAAcgaagCggaAGCU  |
| 2613           | 21 ZBBSR205           | uAc-uu-gUGA-----AACAAcgaagCggaAGCU  |
| 2613           | 22 ZBBSR206           | uAc-uu-gUGA-----AACAAcgaagCggaAGCU  |
| 2613           | 23 ZBBSR235           | uAc-uu-gUGA-----AACAAcgaagCggaAGCU  |
| 2613           | 24 ZBBSR218           | uAc-gc-guC-----UACAAcgcgAggAAGCU  |
| 2613           | 25 ZBBSR217           | uAc-gc-guC-----UACAAcgcgAggAAGCU  |
| 2613           | 26 ZBBSR211           | uAc-gu-GACC-----UACAAcgcgAggAAGCU   |
| 2613           | 27 Pchroma            | uau-cggAUGG-----UGUaccggugggAAGUC   |
| 2613           | 28 EUGROTUN           | uau-cg-gu-----GGAUAgccgauggAAGUu  |
| 2613           | 29 ENTONGING          | ACG-ua-ucAAAUcucuuuuUaacaGuaauAAUUGUGUAUuuUUGAAUauuuUaGuguaUCaaaagAAAGauAAAGAGGCG |
| 2613           | 30 DICDISCO           | uAccugCCU-----CAAgcaggCggaAGUC  |
| 2613           | 31 ZTnucl             | ugg-ug-auA-----CAUAAguuacuggAGGCA   |
| 2613           | 32 W10279             | ugg-ug-auA-----CAUAAguuacuggAGGCA   |
| 2613           | 33 W10274             | ugg-ug-auA-----CAUAAguuacuggAGGCA   |
| 2613           | 34 W10272             | ugg-ug-auA-----CAUAAguuacuggAGGCA   |
| 2613           | 35 W10210             | ugg-ug-auA-----CAUAAguuacuggAGGCA   |
| 2613           | 36 ZBBSR7             | ugg-cu-uug-----CCUaaaUUGcuggAAGUu   |
| 2613           | 37 ZBBSR173           | uag-Au-uug-----CUuaaaaAGcuggAAGUg   |
| 2613           | 38 Siphcyan           | uag-uu-uug-----CCuaaaaAGcuggAAGUu   |
| 2613           | 39 ZBBSR2             | ugg-uu-uug-----UUAaaaUUGcuggAAGUu   |
| 2613           | 40 ZCR4               | ugg-cu-uug-----CAUaaaUUGcuggAGGau   |
| 2613           | 41 ZCR16              | Ugg-cU-uuu-----CAUgaaUCgcuUGAAGUu   |
| 2613           | 42 ZCR6A              | uag-Au-uug-----CUUAAaaaCguggAAGUu   |
| 2613           | 43 PHYPOLYC           | Cag-gg-cCG-----AAUAgguuuUUGAAUu-  |
| 2613           | 44 NAEGRUBE           | uuc-auucGUAA-----ACUAggauggaggAAGau   |
| 2613           | 45 euk heli           | 51  |
| 2613           | 46 rad                | ----->  |

"Sept4aln" on 5-SEP-96 15:33:41

| Posi-<br>tion: | Sequence<br>identity: | Data:    |
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| 2713           | 1                     | 1369mask |
| 2713           | 2                     | MNELEIDY |
| 2713           | 3                     | DIAGRAN  |
| 2713           | 4                     | ATHOMBA  |
| 2713           | 5                     | BLAEMERS |
| 2713           | 6                     | CHLREINH |
| 2713           | 7                     | ORYSATIV |
| 2713           | 8                     | PORAERUG |
| 2713           | 9                     | ACACAST1 |
| 2713           | 10                    | PHRBALAM |
| 2713           | 11                    | STYALSID |
| 2713           | 12                    | EMIHUXLE |
| 2713           | 13                    | OCHDANTC |
| 2713           | 14                    | CAFROENB |
| 2713           | 15                    | LAMBINUT |
| 2713           | 16                    | OXYGRANU |
| 2713           | 17                    | BLEAMERI |
| 2713           | 18                    | HARVERMI |
| 2713           | 19                    | THEANNUL |
| 2713           | 20                    | SYMFILO1 |
| 2713           | 21                    | ZBBSR205 |
| 2713           | 22                    | ZBBSR206 |
| 2713           | 23                    | ZBBSR235 |
| 2713           | 24                    | ZBBSR218 |
| 2713           | 25                    | ZBBSR217 |
| 2713           | 26                    | ZBBSR211 |
| 2713           | 27                    | PchRoma  |
| 2713           | 28                    | EUGROTUN |
| 2713           | 29                    | ENTOTING |
| 2713           | 30                    | DICDISCO |
| 2713           | 31                    | ZTnucl   |
| 2713           | 32                    | W10279   |
| 2713           | 33                    | W10274   |
| 2713           | 34                    | W10272   |
| 2713           | 35                    | W10210   |
| 2713           | 36                    | ZBBSR7   |
| 2713           | 37                    | ZBBSR173 |
| 2713           | 38                    | Siphcyan |
| 2713           | 39                    | ZBBSR2   |
| 2713           | 40                    | ZCR4     |
| 2713           | 41                    | ZCR16    |
| 2713           | 42                    | ZCR6A    |
| 2713           | 43                    | PHYPOLYC |
| 2713           | 44                    | NAEGRUBE |
| 2713           | 45                    | euk hell |
| 2713           | 46                    | rad      |

Position: Sequence Identity:

[illegible]





| Position: | Sequence Identity: | Data:     |
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| 3013      | 4                  | ATHBOMBA  |
| 3013      | 5                  | BLAEMERS  |
| 3013      | 6                  | CHREINH   |
| 3013      | 7                  | ORYSATIV  |
| 3013      | 8                  | PORASERTU |
| 3013      | 9                  | ACACASTI  |
| 3013      | 10                 | PHRBALAM  |
| 3013      | 11                 | STYVALSID |
| 3013      | 12                 | EMIHUKLE  |
| 3013      | 13                 | OCHDANIC  |
| 3013      | 14                 | CAPROENB  |
| 3013      | 15                 | LAMINUT   |
| 3013      | 16                 | ORYGRANU  |
| 3013      | 17                 | BLEAMERI  |
| 3013      | 18                 | HARVERMI  |
| 3013      | 19                 | THEANNUL  |
| 3013      | 20                 | SYMPLOI1  |
| 3013      | 21                 | ZBBSR205  |
| 3013      | 22                 | ZBBSR206  |
| 3013      | 23                 | ZBBSR235  |
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| 3013      | 26                 | ZBBSR211  |
| 3013      | 27                 | Pchroma   |
| 3013      | 28                 | ENTOGUTIN |
| 3013      | 29                 | ENTOGUTIN |
| 3013      | 30                 | DICTDISCO |
| 3013      | 31                 | Ztrnucl   |
| 3013      | 32                 | W10279    |
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| 3013      | 34                 | W10272    |
| 3013      | 35                 | W10210    |
| 3013      | 36                 | ZBBSR17   |
| 3013      | 37                 | ZBBSR173  |
| 3013      | 38                 | Siphycyan |
| 3013      | 39                 | ZBBSR2    |
| 3013      | 40                 | ZCR4      |
| 3013      | 41                 | ZCR16     |
| 3013      | 42                 | ZCR6A     |
| 3013      | 43                 | PHYPOLYC  |
| 3013      | 44                 | NABGRUBE  |
| 3013      | 45                 | euk helli |
| 3013      | 46                 | rad       |

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| Posi-<br>tion: | Sequence<br>identity: | Data:          |
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| 3214 2         | MNELEIDY              | CUUGGAUCC      |
| 3214 3         | DIAGRAN               | C              |
| 3214 4         | ATHBOMBA              | C              |
| 3214 5         | BLAEMERS              | A              |
| 3214 6         | CHLREINH              | G              |
| 3214 7         | ORYSATIV              | G              |
| 3214 8         | PORAERUG              | C              |
| 3214 9         | ACACASTI              | A              |
| 3214 10        | PHRBALAM              | C              |
| 3214 11        | STYALSID              |                |
| 3214 12        | EMIHUXLE              | CUUGGAUCC      |
| 3214 13        | OCHDANIC              | -              |
| 3214 14        | CAFCROENB             |                |
| 3214 15        | LAMINUT               | C              |
| 3214 16        | OXYGRANU              | A-             |
| 3214 17        | BLEAMERI              | C-             |
| 3214 18        | HARVERMI              | C              |
| 3214 19        | THEANNUL              | C              |
| 3214 20        | SYMPILOI              | C              |
| 3214 21        | ZBBSR205              | C              |
| 3214 22        | ZBBSR206              | C              |
| 3214 23        | ZBBSR235              | C              |
| 3214 24        | ZBBSR218              | C              |
| 3214 25        | ZBBSR217              | C              |
| 3214 26        | ZBBSR211              | C              |
| 3214 27        | Pchroma               |                |
| 3214 28        | EUGROTUN              |                |
| 3214 29        | ENTOGING              |                |
| 3214 30        | DICDISCO              | U              |
| 3214 31        | ZTnucl                |                |
| 3214 32        | W10Z79                | C              |
| 3214 33        | W10Z74                | C              |
| 3214 34        | W10Z72                | C              |
| 3214 35        | W10Z10                | C              |
| 3214 36        | ZBBSR7                | C              |
| 3214 37        | ZBBSR173              | C              |
| 3214 38        | Siphcyan              | C              |
| 3214 39        | ZBBSR2                | C              |
| 3214 40        | ZCR4                  | c              |
| 3214 41        | ZCR16                 | C              |
| 3214 42        | ZCR6A                 | C              |
| 3214 43        | PHYPOLYC              | A              |
| 3214 44        | NAEGRUBE              | U              |
| 3214 45        | euk heli              |                |
| 3214 46        | rad                   |                |



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|---|---------------------------------|--|---|
| REPORT DOCUMENTATION<br>PAGE  | 1. REPORT NO.<br>MIT/WHOI 97-08 | 2.   | 3. Recipient's Accession No.                              |
| 4. Title and Subtitle<br>A Molecular Approach to Questions in the Phylogeny of Planktonic Sarcodines  |                                 |  | 5. Report Date<br>September 1996                          |
|   |                                 |  | 6.  |
| 7. Author(s)<br>Linda Angela Amaral Zettler   |                                 |  | 8. Performing Organization Rept. No.                      |
| 9. Performing Organization Name and Address<br>MIT/WHOI Joint Program in Oceanography/Applied Ocean Science & Engineering   |                                 |  | 10. Project/Task/Work Unit No.<br>MIT/WHOI 97-08          |
|   |                                 |  | 11. Contract(C) or Grant(G) No.<br>(C) OCE-9314533<br>(G) |
| 12. Sponsoring Organization Name and Address<br>National Science Foundation; Scurlock Foundation; Bermuda Biological Station for Research, Inc.; Woods Hole Coastal Research Center; the Lerner Gray Fund for Marine Research of the American Museum of Natural History.  |                                 |  | 13. Type of Report & Period Covered<br>Ph.D. Thesis       |
|   |                                 |  | 14.   |
| 15. Supplementary Notes<br>This thesis should be cited as: Linda Angela Amaral Zettler, 1996. A Molecular Approach to Questions in the Phylogeny of Planktonic Sarcodines. Ph.D. Thesis. MIT/WHOI, 97-08.   |                                 |  |   |
| 16. Abstract (Limit: 200 words)<br>The relationships of the Acantharea and the Polycystinea (order Spumellarida) to other protists were investigated using phylogenetic analyses of small-subunit ribosomal RNA (SSU rRNA) genes. These two classes are included in the superclass Actinopoda based on specialized pseudopodia called axopodia. SSU rRNA-based phylogenies do not support the common ancestry of the Acantharea and the Polycystinea, implying that the superclass Actinopoda is artificial and should be discarded. All analyses supported the monophyly of the Acantharea and the separate monophyly of the Polycystinea. The origin of the sequences was confirmed by <i>in situ</i> hybridizations.<br>The SSU rRNA-based method was also useful in fine-scaled comparisons. Sequence comparisons for the solitary spumellarian <i>Thalassicolla nucleata</i> from the Sargasso Sea and the Pacific Ocean indicate there may be different species which are morphologically identical or that there may be allelic variation in a given individual. Analyses between the solitary <i>T. nucleata</i> and seven colonial spumellaria indicate that the colonial spumellaria may not be monophyletic. The results from examination of branching patterns within three of the four orders of the Acantharea placed the symphyacanthid <i>Haliommatidium</i> sp. branching among the Arthracanthida, indicating that the Symphyacanthida are not monophyletic as defined by morphology-based systematics. |                                 |  |   |
| 17. Document Analysis    a. Descriptors<br>Radiolaria<br>Acantharia<br>Polycystinea<br><br>b. Identifiers/Open-Ended Terms<br><br><br>c. COSATI Field/Group   |                                 |  |   |
| 18. Availability Statement<br>Approved for publication; distribution unlimited.   |                                 | 19. Security Class (This Report)<br>UNCLASSIFIED | 21. No. of Pages<br>200                                   |
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